REGULATION OF *CHOP* TRANSLATION IN RESPONSE TO eIF2 PHOSPHORYLATION AND ITS ROLE IN CELL FATE

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DEDICATION

I dedicate my thesis to my parents Sivarami Reddy Palam and Jayalakshmamma Palam, who inspired me and gave me the courage to pursue my graduate school aspirations. I am grateful for their tremendous support.

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I am greatly indebted to my mentor Dr. Ronald Wek for his valuable advice, support, patience, and encouragement during my graduate career. I hope for similar support in the future. I thank my committee members Dr. Robert Harris, Dr. David Skalnik, and Dr. Paul Herring for their valuable time and advice over the years. I am grateful to Sheree Wek for her help, and my fellow lab members and friends Souvik Dey, Tom Baird, and Brian Teske for technical help, support, and suggestions from our many conversations. I extend my thanks to Dr. Wek's former students Dr. Kirk Staschke and Dr. Dongui Zhou. Dr. Ivanov (University of Utah) kindly provided reagents which were useful for my graduate studies. I also thank Dr. Howard Edenberg, Dr. Yunlong Liu, and Dr. Jeanette McClintick for their assistance with my microarray analysis. Lastly, I sincerely thank my wife Sreelatha Siripi for her support, encouragement, and understanding.

ABSTRACT

Lakshmi Reddy Palam

REGULATION OF *CHOP* TRANSLATION IN RESPONE TO eIF2 PHOSPHORYLATION AND ITS ROLE IN CELL FATE

In response to different environmental stresses, phosphorylation of eukaryotic initiation factor-2 (eIF2) rapidly reduces protein synthesis, which lowers energy expenditure and facilitates reprogramming of gene expression to remediate stress damage. Central to the changes in gene expression, eIF2 phosphorylation also enhances translation of ATF4, a transcriptional activator of genes subject to the Integrated Stress Response (ISR). The ISR increases the expression of genes important for alleviating stress, or alternatively triggering apoptosis. One ISR target gene encodes the transcriptional regulator CHOP whose accumulation is critical for stress-induced apoptosis. In this dissertation research, I show that eIF2 phosphorylation induces preferential translation of *CHOP* by a mechanism involving a single upstream ORF (uORF) located in the 5'-leader of the *CHOP* mRNA. In the absence of stress and low eIF2 phosphorylation, translation of the uORF serves as a barrier that prevents translation of the downstream *CHOP* coding region. Enhanced eIF2 phosphorylation during stress facilitates ribosome bypass of the uORF, and instead results in the translation of *CHOP*. Stable cell lines were also constructed that express *CHOP* transcript containing the wild type uORF or deleted for the uORF and each were analyzed for expression changes in response to the different stress conditions. Increased CHOP levels due to the absence of inhibitory uORF sensitized the cells to stress-induced apoptosis when compared to the

cells that express *CHOP* mRNA containing the wild type uORF. This new mechanism of translational control explains how expression of *CHOP* and the fate of cells are tightly linked to the levels of phosphorylated eIF2 and stress damage.

Ronald C. Wek, Ph.D., Chair

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ABBREVIATIONS

ASNS	asparagine synthase
ATF	activating transcription factor
ATF3	activating transcription factor 3
ATF4	activating transcription factor 4
ATF5	activating transcription factor 5
ATF6	activating transcription factor 6
bZIP	basic zipper
C/EBP	CCAAT enhancer binding portein
CHOP	C/EBP homologous protein
CReP	constitutive repressor of eIF2~P
C-terminus	carboxy terminus
DsRBM	double-stranded RNA-binding motif
DTT	dithiothreitol
ERSE	ER stress response element
EBER	Epstein-Barr Virus Small RNA
eIF	eukaryotic initiation factor
eIF2	eukaryotic initiation factor-2
eIF2B	eukaryotic initiation factor B
ER	endoplasmic reticulum
GAAC	general amino acid control
GADD34	growth arrest and DNA damage-inducible protein 34
GCN	general control nonderepressible

GCN2	general control nonderepressible 2
GDI	guanosine diphosphate dissociation inhibitor
GEF	guanine nucleotide exchange factor
HisRS	histidyl-tRNA synthetase
HIV-1	human immunodeficiency virus type 1
HRI	heme-regulated inhibitor
IFN	interferon
IPTG	isopropyl β-D-1-thiogalactopyranoside
ISR	integrated stress response
Met-tRNAi	methione- initiator methionyl-tRNA
Min	minute(s)
mRNA	messenger RNA
mTOR	mammalian target-of-rapamycin
NaF	sodium fluoride
NF-κB	nuclear factor kappa B
PCR	polymerase chain reaction
PEK	pancreatic eIF2 kinase
PERK	PKR-like ER kinase
PKR	double-stranded RNA-activated kinase
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase 1
PP1c	catalytic subunit of protein phosphatase 1
qRT	quantitative reverse transcription

RT	reverse transcriptase
S.D.	standard deviation
S.E.	standard error
SLIC	sequence and ligase independent cloning
SS	signal sequence
TC	ternary complex
TF	transcription factor
TOR	target-of-rapamycin
TM	transmembrane
uORF	upstream open reading frame
UPR	unfolded protein response
UTR	untranslated region
WRS	Wolcott-Rallison Syndrome

INTRODUCTION

1. Mechanisms regulating protein synthesis in response to environmental stresses

Rapid changes in global and gene-specific translation occur in response to many different environmental stresses. For example, translation is repressed when there is accumulation of misfolded protein in the endoplasmic reticulum, which prevents further overload of the secretory pathway and provides time for reconfiguration of gene expression with a focus on stress alleviation (1, 2). A central mechanism for this translational control involves phosphorylation of eukaryotic initiation factor 2 (eIF2~P) by the double-stranded RNA activated protein kinase (PKR) like ER kinase (PERK) or pancreatic eIF2 kinase (PEK) (3, 4). eIF2 is a translation initiation factor that combines with initiator Met-tRNAi^{Met} and GTP and participates in the selection of the start codon. Phosphorylation of the α subunit of eIF2 at Ser-51 in response to endoplasmic reticulum (ER) stress blocks the exchange of eIF2-GDP to eIF2-GTP, thus reducing global translation initiation and subsequent protein synthesis (5, 6).

In addition to PERK, three other eIF2 kinases respond to other stress conditions, including general control nonderepressible 2 (GCN2) induced by nutritional deprivation, heme-regulated inhibitor (HRI) activated by heme deficiency in erythroid cells, and PKR which functions in an anti-viral defense pathway (4, 5). Accompanying this repression of global translational initiation, eIF2~P selectively enhances the translation of *ATF4* mRNA, encoding a basic zipper (bZIP) transcriptional activator of stress-related genes involved in metabolism, protection against oxidative damage, and regulation of apoptosis (1, 3, 7-9). The idea that ATF4 is a common downstream target that integrates signaling from PERK and other eIF2 kinases has led to the eIF2~P/ATF4 pathway being

collectively referred to as the Integrated Stress Response (ISR) (10). Elevated ATF4 levels induce additional bZIP transcriptiona l regulators, such as CHOP and ATF3, which together direct a program of gene expression important for cellular remediation, or alternatively apoptosis (9-11). Deregulation of eIF2 kinase pathways may lead to disease complications (1-3, 5, 12-14).

2. Multiple translation factors facilitate translation initiation

The eIF2 consists of three subunits (α,β, and γ) and binds with GTP and initiator Met-$tRNA_i^{Met}$ during translation initiation (5, 6). The so-called eIF2 ternary complex associates with the 40S ribosomal subunit, resulting in a 43S pre-initiation complex that is also joined with additional translation initiation factors, eIF1, eIF1A, eIF3 and eIF5 (5, 6). The 43S complex then localizes to the cap structure and associated eIF4F proteins situated at the 5'-end of target mRNAs. Upon binding to the cap structure, the 43S ribosome scans in 5' to 3' direction along the 5'-leader of the mRNA, searching for an initiation codon. This is typically the first AUG codon, and selection can be enhanced by an optimum sequence context -GCC(*A/G*$^{-3}$)CCA**AUG***G*$^{+4}$-, with the initiation codon in underline and bold and a flanking purine at the -3 and a G at the +4 positions (15). Together the eIF1 and eIF1A facilitate the recognition and selection of initiating codons. eIF1 plays a key role in the fidelity of AUG selection by preventing translation initiation at non-AUG codons (16, 17). Conformational changes in 43S complex accelerate the GTPase activity of eIF5 that facilitates the eIF2-GTP hydrolysis to eIF2-GDP and inorganic phosphate (18). The irreversible eIF2-GTP hydrolysis occurs only when Pi

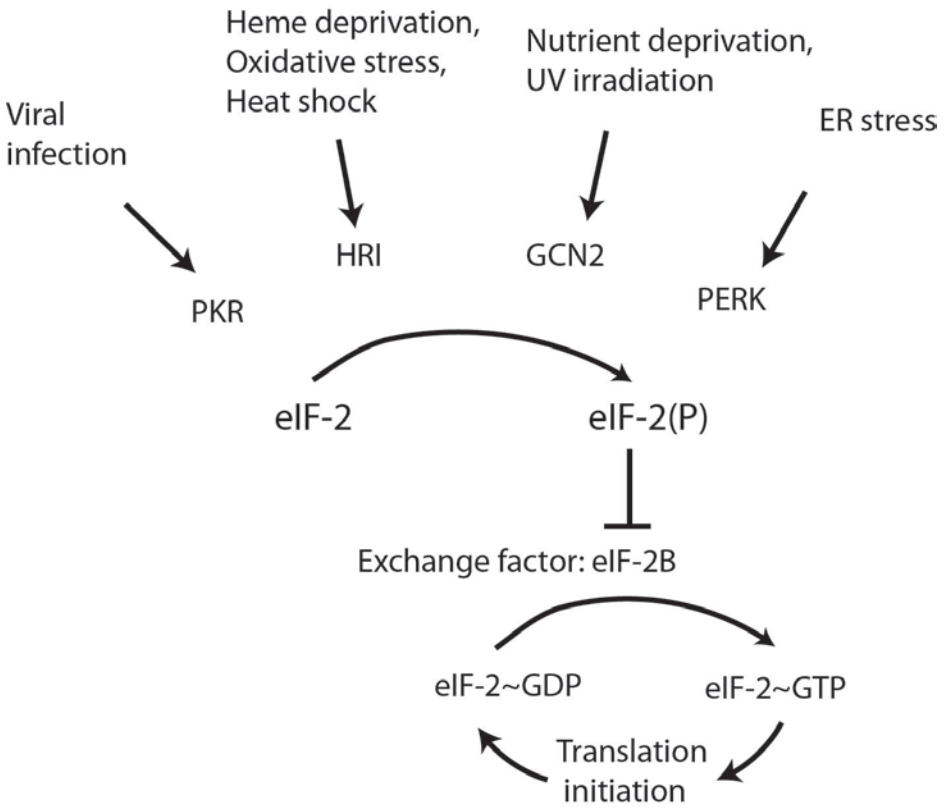


Figure 1. Diverse stress conditions activate family of eIF2 kinases and phosphorylate eIF2*α* at serine 51. Protein kinases PKR, HRI, GCN2, and PERK, each respond to different stress conditions and phosphorylate eIF2. Phosphorylated eIF2 acts as competitive inhibitor to eIF2B, a guanine nucleotide exchange factor that is required for conversion of eIF2-GDP to eIF2-GTP. The resulting lowered levels of eIF2-GTP repress global translation initiation.

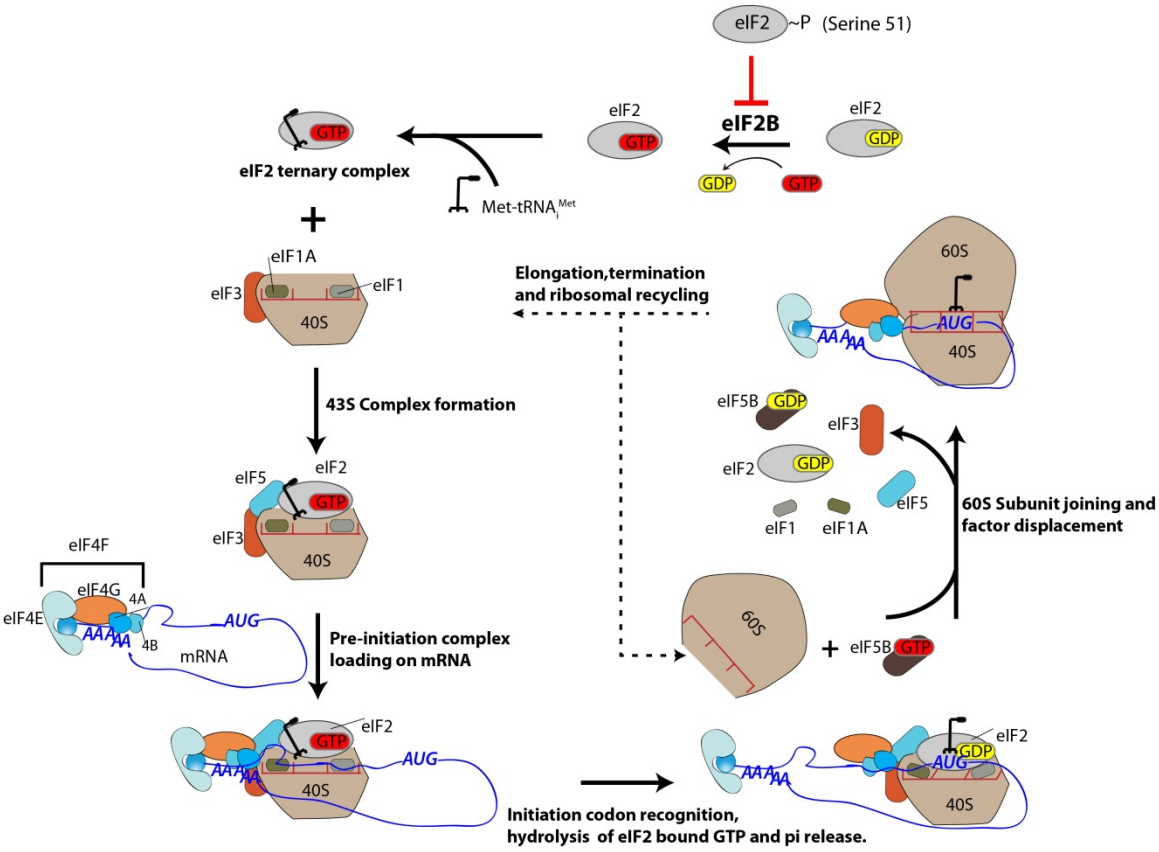


Figure 2. eIF2 in association with GTP and Met-tRNA$_i^{Met}$ participates in translation initiation. eIF2 forms a ternary complex with GTP and Met-tRNA$_i^{Met}$, and facilitates joining of the initiator tRNA to the 40S ribosomal subunit. The 40S ribosomal subunit with the ternary complex forms a 43S pre-initiation complex together with other initiation factors eIF1, eIF1A, and eIF5. eIF4F facilitates loading of the 43S complex to 5'-cap of mRNAs consisting of a 7'-methyl guanosine. With the help of eIF3 and the RNA helicase eIF4A, the 43S complex progressively scans in 5' to 3' direction along the 5' leader of the mRNA in search of an initiation codon. The GTPase function of eIF5 facilitates the eIF2-GTP hydrolysis to eIF2-GDP and Pi. Upon recognition of the initiator AUG in the P site of the 43S complex, the Pi is released from eIF2. Following the dissociation of eIF2-GDP, the 60S ribosomal subunit combines with the 40S ribosomal subunit to form the

80S complex, and translation elongation begins. A family of eIF2 kinases phosphorylates the α subunit of eIF2 at serine 51 in response to various stress stimuli. Phosphorylated eIF2 itself becomes a competitive inhibitor to the guanine nucleotide exchange factor, eIF2B, which is required for recycling of eIF2-GDP to eIF2-GTP. The decrease in eIF2-GTP levels during eIF2 phosphorylation results in reduced translation initiation. Lowered protein synthesis allows cells sufficient time to remedy the stress damage. A program of gene expression is also initiated in response to stress induced eIF2~P, which allows cells to adapt to the stress conditions.

releases from eIF2, which occurs upon base pairing between the anticodon of $tRNA_i^{Met}$ and the initiation codon of the mRNA. The release of Pi from eIF2 is regulated by dissociation of eIF1 from the 43S/mRNA complex (19, 20). With release of eIF2-GDP, eIF5B facilitates the 60S ribosomal subunit joining to the 40S subunit to form the 80S ribosomal complex. Translation elongation then begins by accepting aminoacyl-tRNAs into the A (aminoacyl) site of the ribosome for subsequent formation of peptide bonds (6).

3. eIF2B facilitates eIF2-GTP exchange that is inhibited by phosphorylated eIF2

Eukaryotic initiation factor 2B (eIF2B) is the guanine nucleotide exchange factor (GEF) for eIF2 that recycles eIF2 associated with GDP to eIF2-GTP. eIF2B is a heteropentameric complex that consists of 5 subunits, designated α, β, γ, δ and ε in mammals, and the yeast counterparts Gcn3p, Gcd7p, Gcd1p, Gcd2p, and Gcd6p, respectively (6, 21, 22). The γ and ε subunits facilitate the catalytic function of eIF2B, while the α, β, and δ subunits serve a regulatory function (23-26). Phosphorylation of the α subunit of eIF2 at serine 51 in response to various stresses alters the initiation factor from a substrate to a competitive inhibitor of eIF2B, associating with the regulatory portion of eIF2B and blocking exchange of eIF2-GDP to eIF2-GTP (5, 6).

Recent studies indicate that the yeast eIF5 can control the eIF2-GTP levels through its novel GDP dissociation inhibitor (GDI) function (27). eIF5 binds to eIF2-GDP by its carboxyl terminal domain, and sequesters available eIF2 from eIF2B, thus reducing the exchange to eIF2-GTP. Furthermore, eIF5 was reported to have a high

affinity for eIF2 when its α subunit is phosphorylated, suggesting that eIF5 (GDI) can assist in the regulation of eIF2 in response to stress conditions (27, 28).

4. Feedback regulation by eIF2 dephosphorylation

Cells reduce translation and conserve energy resources through eIF2~P during diverse stress conditions. Dephosphorylation of eIF2 is required for resumption of general protein synthesis. The first identified phosphatase complex dephosphorylating eIF2~P consisted of cellular catalytic subunit protein phosphatase-1 (PP1c) and a viral regulatory subunit encoded by the herpes simplex virus gene $\gamma_1$34.5 (29). By dephosphorylating eIF2, herpes virus escapes the antiviral effects of PKR. Growth arrest and DNA damage -34 (GADD34) is a cellular homolog of $\gamma_1$34.5 that recruits type 1 serine/threonine protein phosphatase 1 specifically to eIF2 (30-33). GADD34 is not readily detectable in normal cells but is transcriptionally up regulated by ATF4 in response to stress (34, 35). This feedback mechanism facilitates resumption of general protein synthesis. Constitutive Repressor of eIF2~P (CReP) is another well-studied protein that specifically recruits PP1 to phosphorylated eIF2 (36). Unlike GADD34, CReP is constitutively expressed in cells. Mice deleted for CReP survive gestation, but exhibit severe growth retardation and impaired erythropoiesis (37). Deletion of both *CReP* and *GADD34* in mice leads to embryonic lethality, indicating that proper regulation of eIF2~P is important for developmental processes (37).

5. Different mechanisms activate the eIF2 kinases

Each of the eIF2 kinases are activated by different stresses. PERK is induced in response to accumulation of unfolded protein in the ER (1-3). PERK is a transmembrane protein with its regulatory region in the lumen of the ER and a cytosolic protein kinase domain. BiP is a molecular chaperone present in the ER that is reported to bind to the PERK luminal domain in the absence of stress. Upon stress induction, BiP dissociates from PERK, allowing PERK dimerization (38, 39). PERK dimerization is suggested to lead to a conformational change that contributes to autophosphorylation in the kinase activation loop of PERK, which leads to enhanced eIF2~P. PERK phosphorylation of eIF2 increases the expression of ATF4, which then contributes to activation of a cascade of transcription factors, including ATF3 and CHOP (4, 11, 40, 41). This model is supported by studies showing that the fusion of a dimerization domain to the PERK kinase domain leads to activation of this eIF2 kinase in the absence of stress (42). Furthermore, PERK inactivation occurs by deletion of a dimerization region from amino acid residues 102 to 407 in PERK (38).

In addition to nutrient deprivation, GCN2 can be activated by UV irradiation and proteasome inhibition (43-45). Central to the regulation of GCN2 is a region homologous to histidyl-tRNA synthetase enzymes, referred to as the HisRS-related domain. The mechanism of GCN2 activation involves binding of uncharged tRNA that accumulates during amino acid limitation to the HisRS-related regulatory domain (22, 46-50). Uncharged tRNAs binding to the HisRS region is suggested to cause conformational changes in GCN2, which facilitates GCN2 autophosphorylation at sequences in the protein kinase activation loop (47, 49). In response to UV irradiation, GCN2

phosphorylates eIF2 and reduces protein synthesis. Lowered protein synthesis diminishes the levels of the labile IκBα protein, which functions as an inhibitor of the transcription factor NF-κB (43). Thus GCN2 confers resistance to apoptosis in response to UV irradiation through activation of NF-κB and induced expression of its target genes (43).

ATF4 is differentially regulated in response to various stresses. Even though robust eIF2~P occurs in response to UV irradiation, ATF4 synthesis is hampered. The underlying reason for the uncoupling between eIF2~P and induced ATF4 synthesis is that *ATF4* transcription is repressed during UV irradiation (51). Therefore, there are only low levels of *ATF4* mRNA, which cannot be readily translated in response to eIF2~P. Forced expression of ATF4 by salubrinal pretreatment followed by UV irradiation suggests that elevated levels of ATF4 during UV stress is detrimental to cell survival (51).

The eIF2 kinase PKR participates in an anti-viral defense mechanism that is mediated by interferon (IFN) (52-54). PKR contains two double-stranded RNA-binding motifs (dsRBMs) upstream of its protein kinase domain, which are central for induced eIF2~P (52-54). Double-stranded RNAs which can accumulate during many different viral infections is suggested to bind to the dsRBMs, facilitating a bridge between PKR polypeptides, which triggers PKR autophosphorylation and an activated eIF2 kinase (55). Interferons α and β that are produced during viral infection further induce this mode of translational control by increasing the transcription of *PKR*. The eIF2~P in turn reduces cellular mRNA and viral mRNA translation, thus limiting viral proliferation (55).

Viruses can mitigate the PKR-defense system by producing RNAs or proteins that directly or indirectly alter PKR activity (55-57). For example, the NS5A protein from hepatitis virus was reported to directly bind to PKR and inactivate the eIF2 kinase (58).

Vaccinia virus protein K3L mimics the substrate eIF2α, thus acting as substrate decoy that binds to and blocks the PKR catalytic pocket (59). The E3L from vaccinia virus and NS1 from influenza virus are proteins with dsRBMs that are proposed to bind and sequester the dsRNA, thus precluding PKR activation (60). In the case of herpes virus, as discussed above, the protein $\gamma_1$34.5 recruits PP1c to dephosphorylate eIF2~P, and thereby avoid PKR repression of translation (61). Along with eIF2~P regulation, PKR was shown to function in a variety of signal transduction pathways, including those involving interleukin-3, NF-κB, p53, interferon regulatory factor-1, platelet-derived growth factor, IFN-β, STAT1, and mitogen-activated protein kinases (62). These pathways can affect cell survival, with PKR being suggested to trigger apoptosis as part of the strategy to thwart viral infection and proliferation.

HRI is expressed predominantly in erythroid cells. HRI is regulated by heme through the two heme-binding regions in HRI: an N-terminal domain of HRI and in an insert region in the protein kinase domain of HRI (63). Heme, in the presence of iron, binds to α and β globin chains in ratio of 1:2:2, respectively. In response to iron deficiency, conformational changes in heme cause a release from the kinase insert portion of HRI, allowing for HRI autophosphorylation and activation to occur (63, 64). The activated HRI then phosphorylates eIF2 and inhibits protein synthesis. Therefore, globin protein synthesis is reduced during heme deprivation and the balance between the levels of globin protein are retained with respect to available iron and heme content. *HRI*$^{-/-}$ mice show high globin content despite iron depletion, resulting in globin aggregation and

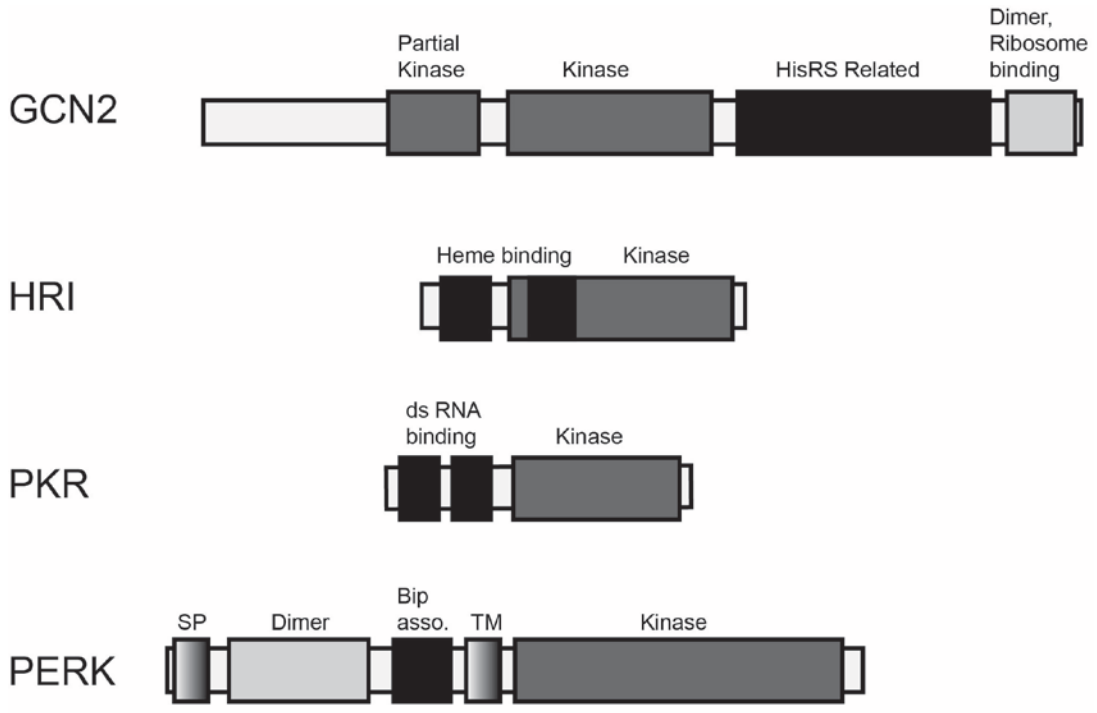


Figure 3. eIF2 Kinases, GCN2, HRI, PKR, and PERK regulate translation in response to different stresses. Each eIF2 kinase has a conserved protein kinase domain and distinct regulatory domains that serve to recognize different stress conditions. In response to amino acid starvation, accumulated uncharged tRNAs bind to the HisRS-related domain of GCN2 causing conformational changes that facilitates activation of the protein kinases. The carboxyl terminal region allows for GCN2 dimerization and also for this eIF2 kinase to associate with ribosomes. Heme binds to amino-terminal sequences of HRI, along with a kinase insert region, leading to inhibition of eIF2 kinase activity. In response to iron deficiency in erythrocytes, heme is released from HRI, facilitating phosphorylation of eIF2. During viral infection, accumulated double-stranded RNA binds to two dsRBMs, facilitating a conformation change that enhance PKR autophosphorylation and increase the phosphorylation of eIF2. PERK exists as a

transmembrane protein in the ER. The regulatory luminal portion of PERK associates with ER chaperones, such as BiP. The dimerization domain and ER transmembrane (TM) region are important for PERK proximity to the ER and activation of the eIF2 kinase. During the unfolded protein response, BiP dissociates form the luminal portion of PERK, allowing PERK dimerization and induced protein kinase function. PERK phosphorylation of eIF2 represses global translation and initiates a program of gene expression that is designed to reduce the influx of newly synthesized proteins into the stressed ER.

enhanced apoptosis of erythroid precursors in bone marrow and spleen that is characterized by hyperchromia and compensatory erythroid hyperplasia (63, 64).

In response to various stress conditions, eIF2~P represses global translation coincident with enhanced translation of a specific set of mRNAs, such as those encoding the bZIP transcription factors GCN4 in yeast and ATF4 and ATF5 in mammals (5, 66). The upstream open reading frames (uORF) present in the 5'-leader of these mRNAs regulate their translation in response to eIF2~P. In response to amino acid depletion, enhanced GCN4 protein in yeast triggers the expression of genes involved in amino acid biosynthesis and the uptake and salvaging of nutrients (67). In mammals, the related ATF4 is induced in response to a broader spectrum of stresses, leading to activation of genes involves in metabolism, the redox status of cells, and the regulation of apoptosis (10).

6. Mechanisms underlying gene-specific translation in response to eIF2~P

Translational expression of *GCN4* involves four uORFs in the 5' leader of the *GCN4* mRNA. These uORFs are only two to three codons in length. The uORFs facilitate *GCN4* translation control by a mechanism involving three features (22). First, the translation initiation complex with eIF2/GTP/Met-tRNA$_i^{Met}$ processively scans the 5'-leader of the *GCN4* mRNA and initiates translation at the 5'-proximal uORF1. Second, after translation of uORF1, the post-terminating ribosomes are proposed to retain association with the *GCN4* mRNA (68). More than 50% of ribosomes are thought to resume scanning along the 5'-leader of the *GCN4* transcript (69-71). The rationale for why reinitation can occur following translation of the uORF1 is not fully understood. An

A+U-rich region around the uORF1 stop codon is critical for retaining re-initiating ribosomes that resume mRNA scanning in search of downstream start codon (72). Recent studies on eukaryotic initiation factor 3 (eIF3) showed that sequences 5' to the uORF1 interact with the N-terminal domain of eIF3 subunit a (eIF3a), a critical factor for ribosome reinitiation (73, 74). Furthermore, a mechanism was proposed that the 5' cis-acting elements or reinitiation-promoting elements (RPEs) preceding the short uORFs progressively fold and interact with eIF3a, and facilitate translation reinitiation (71). The third feature of the GCN2 translation control model involves the timing of reinitiation based on the availability of eIF2-GTP (22, 68, 69).

In the presence of high eIF2-GTP levels, the scanning ribosomes reinitiate translation more rapidly at uORF2, uORF3 or uORF4. Translation of these inhibitory uORFs leads to dissociation of the ribosomes from the *GCN4* mRNA, and therefore low synthesis of GCN4 (22, 68, 69). During stress conditions such as amino acid starvation, GCN2 phosphorylates eIF2. The resulting lower levels of eIF2-GTP cause a delay in the scanning ribosomes to reacquire the eIF2 ternary complex, which allows for the bypass of negative-acting uORF2, uORF3 and uORF4. During the interval between the uORF4 and the *GCN4* coding region, scanning ribosomes would reacquire the eIF2 ternary complex and recognize the *GCN4* initiation codon. Elevated GCN4 protein levels would then contribute to a program of gene expression that adapts to nutrient deficiency (67, 75).

This model for *GCN4* translational control is supported by a wealth of genetic and biochemical studies. For example, fusion of the 5'-leader of the *GCN4* mRNA to a *lacZ* reporter is sufficient to confer translational control by eIF2~P (76, 77). Deletion of the

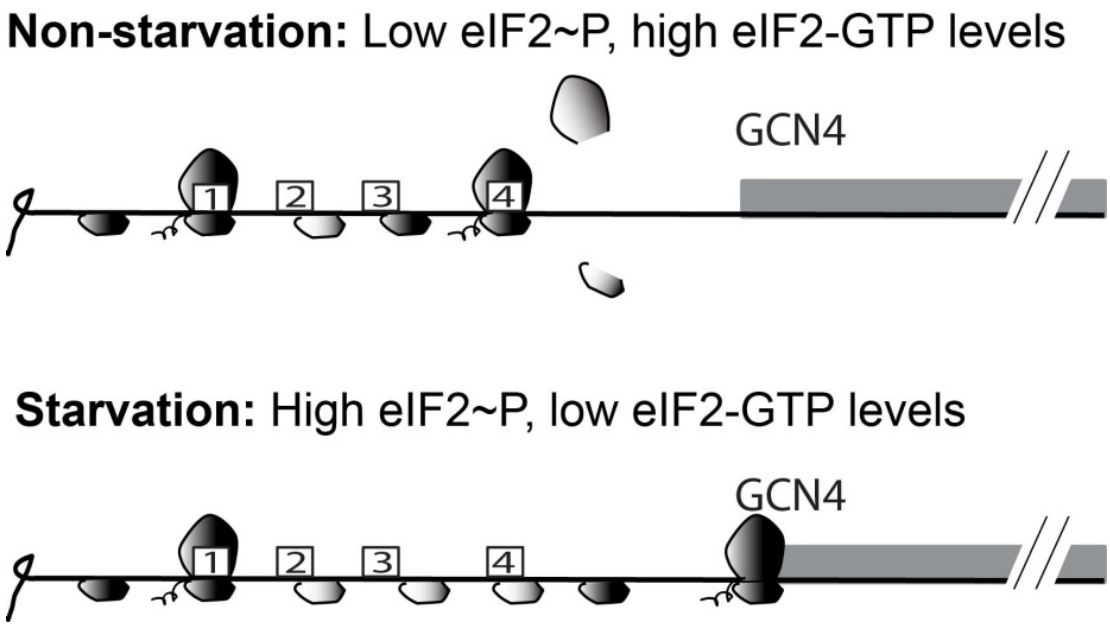


Figure 4. Amino acid starvation induces eIF2 phosphorylation and *GCN4* translation. The yeast *GCN4* mRNA has four short uORFs in its 5' leader that serve to direct preferential translation in response to eIF2~P. uORF1 acts as positive element in the translation control, and uORF2, uORF3 and uORF4 function as inhibitors of *GCN4* expression. The 43S preinitiation complex binds to the 5'-cap structure and scans mRNA in 5' to 3' direction, initiating translation at the 5'- proximal uORF1. After translation of the uORF1, more than 50% of terminating ribosomes retain association with the mRNA and resume scanning to reinitiate translation at a downstream ORF. Under non-stressed conditions, the scanning ribosomes quickly reacquire the eIF2 ternary complex and reinitiate translation at uORF2, uORF3, or uORF4. Following translation of one of these inhibitory uORFS, ribosomes dissociate from the *GCN4* transcript. Thus *GCN4* translation is repressed during high eIF2-GTP levels when there are only low amounts of eIF2~P. During amino acid starvation, there is induced eIF2~P and low eIF2-GTP levels.

This causes a delay in the delivery of the eIF2 ternary complex to ribosomes that have recently completed translation of uORF1 and resumed scanning along the 5'-leader of the *GCN4* mRNA. This delay in ribosome reinitiation allows the bypass of the inhibitory uORFs 2-4, and instead ribosomes translate the *GCN4* coding region. Increased GCN4 protein directly triggers the transcription of a collection of genes that are required for alleviation of nutrient deficiencies.

positive-acting uORF1 blocks *GCN4* translation because there is an absence of ribosome reinitiation (77). By comparison deletion of the inhibitory uORFs 2-4 result in high levels of *GCN4* translation independent of eIF2~P. In fact, the presence of the uORF1 and a single inhibitory uORF4 are sufficient for eIF2~P-mediated control of *GCN4* translation (77). This finding is germane to the translation regulation of *ATF4*, which will be discussed later. The role of eIF2~P and its attendant reduced eIF2B function is supported by several genetic studies (78). For example, abolishing eIF2~P, by substituting serine 51 to alanine in eIF2α (yeast *SUI2*), prevents a reduction in eIF2-GTP levels and constitutive repression of GCN4 translation (a so-called Gcn$^-$ phenotype) (68). By comparison, missense mutations in GCD1 (γ subunit of eIF2B), which reduce eIF2B activity independent of eIF2~P lead to high levels of *GCN4* translation independent of eIF2~P (so-called Gcd$^-$ phenotype) (22).

The mechanism underlying *ATF4* mRNA translational control shares critical features with the *GCN4* translational mechanism (7). The 5'-leader of the *ATF4* mRNA contains two uORFs. The uORF1 expresses a polypeptide only 3 amino acid residues in length, whereas the uORF2 is 59 amino acid residues in length. The uORF2 overlaps out-of-frame with the *ATF4* coding region by 83 nucleotides. These uORFs participate in the *ATF4* translational control by a mechanism that is similar to that described for *GCN4*. The ribosome initiation complex, which includes the eIF2 ternary complex, initiates translation at the 5'-proximal uORF1. After translation of uORF1, a portion of the terminating ribosomes retain the capacity to reinitiate translation at the downstream region. In conditions devoid of stress there are high eIF2-GTP levels, allowing for ribosomes to quickly reacquire the eIF2 ternary complex and reinitiate translation at

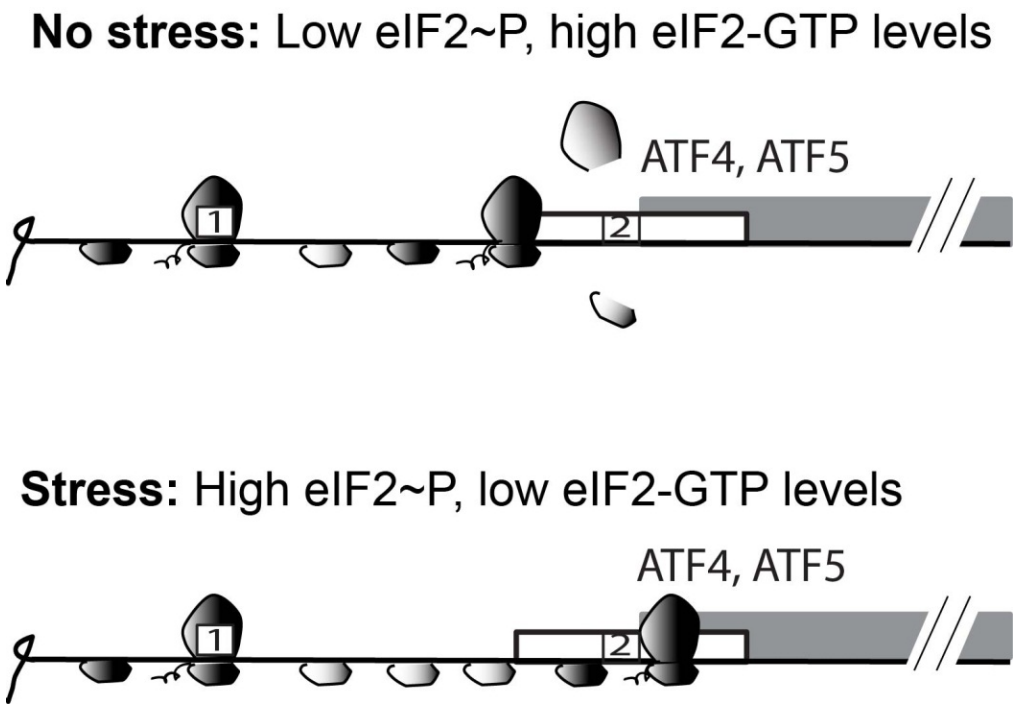


Figure 5. Regulation of *ATF4* and *ATF5* mRNA translation in response stress and induced eIF2 phosphorylation. *ATF4* mRNA has two uORFs: a short uORF1 that is three codons in length and a longer uORF2, which is 183 nucleotides in length and overlaps 83 nucleotides out-of-frame with the *ATF4* coding region. Ribosomes begin translating the *ATF4* mRNA at the 5'-proximal uORF1. Following translation of uORF1, a portion of the ribosomes retain the capacity to reinitiate translation at a downstream ORF. During non-stressed conditions there are high eIF2-GTP levels, allowing for scanning ribosomes to quickly reacquire the eIF2 ternary complex and initiate translation at the inhibitory uORF2. As uORF2 is out-of-frame with the *ATF4* coding region, translation of uORF2 precludes translation of the *ATF4* coding region. Hence there is low ATF4 synthesis. Under stress conditions with low eIF2-GTP levels due to eIF2~P, there is a delay in delivery of eIF2 ternary complex to the reinitiating ribosomes. This delay in ribosome reinitiation allows the scanning ribosomes to bypass the inhibitory uORF2.

Instead translation initiation occurs downstream at the *ATF4* coding region. The regulation of *ATF5* mRNA translation regulation in response to eIF2~P also involves this ribosome reinitiation mechanism (66).

uORF2 (79). As uORF2 overlaps with the initiation codon of the *ATF4* coding region, translation of uORF2 precludes the translation initiation at the downstream *ATF4* coding region. During stress conditions, enhanced eIF2~P would reduce the eIF2-GTP levels. Following translation of uORF1, the scanning ribosomes would be delayed for reinitiation, allowing the bypass of the inhibitory uORF2. However, during the interval between the initiation codons of the uORF2 and *ATF4* coding regions, the scanning ribosomes re-attain the ternary complex, and translate the *ATF4*. Enhanced ATF4 protein would then directly activate the transcription of target genes involved in adaptation to stress.

ATF5 is another well studied gene that is translationally regulated in response to eIF2~P. ATF5 is induced in response to diverse stress conditions and is transcriptionally enhanced by ATF4 in response to eIF2~P (66). The 5'-leader of the *ATF5* mRNA has two uORFs, which are organized similar to the *ATF4* transcript. The start codon context of the uORF1 in *ATF4* and *ATF5* mRNAs share a strong consensus with the so-called Kozak sequence (5'-GCCACC**AUG**G-3') (66, 79). Also, uORF1 coding region from both *ATF4* and *ATF5* are only 3 codons in length. These two features are highly conserved among different species. This suggests that the efficient initiation at uORF1 and length of the uORF1 are critical for the positive-acting roles in *ATF4* and *ATF5* mRNA translation. Genetic analyses of this leader structure support the idea that the underlying mechanism of translation control in response to eIF2~P also involves a delayed ribosome reinitiation mechanism similar to that of *ATF4*. ATF5 is suggested to have a pro-apoptotic role in response to certain stresses (80). However, ATF5 in glioblastomas is reported to be critical for survival of cancer cells (81, 82). Additionally, ATF5 is reported to be

important in neuronal cell differentiation (83). This suggests that ATF5 may have many different biological functions depending on the developmental stage and cell types.

7. Additional regulators of the ISR are subject to translational control

During the ISR, ATF4 enhances the transcription of *GADD34*. *GADD34* mRNA was shown to be preferentially associated with large polysomes in response to eIF2~P, analogous to *ATF4* and *ATF5* mRNAs (84). *GADD34* mRNA also has two uORFs in its 5'-leader, however the uORF arrangement is different from *ATF4* or *ATF5* (84). The uORF1 and uORF2 are overlapping in the mouse *GADD34* transcript, whereas in human *GADD34*, the uORF1 is separated by 30 nucleotides from uORF2. Both uORFs can inhibit the downstream *GADD34* coding region translation during normal conditions. uORF1 has only a moderate repression, whereas uORF2 strongly inhibits the translation of the *GADD34* coding region. A recent study suggests that ribosomes may proceed through the inhibitory uORFs in response to stress induced eIF2~P, but the mechanism of the bypass is not clear (84). Given the importance of GADD34 in the eIF2~P feedback mechanism, the regulation of *GADD34* translation is critical for cell adaptation to stress, or alternatively apoptosis.

In response to various stresses, *CHOP* is transcriptionally induced by bZIP transcription factors, such as ATF4 and ATF5 (34, 35). During hypoxic conditions, *CHOP* mRNA was suggested to be associated with large polysomes, indicating that *CHOP* may also be subject to translation control in response to stress (85). ATF4-directed transcriptional regulation of *CHOP* is well characterized, but the possible mechanisms involved in *CHOP* translational regulation are not yet known. This will be a

central topic of this thesis. The levels of CHOP protein are critical for determining cell adaptation to stress conditions (86). In response to chronic stress, CHOP promotes cellular apoptosis. Also, CHOP heterodimerizes with other bZIP transcription factors, such as ATF4, and can regulate the expression of ISR genes, including *GADD34* that is important for feedback control of the ISR (34, 35). Given the significance of *CHOP* function in cellular stress responses, comprehensive studies are much needed to understand the mechanisms regulating *CHOP* translation. This topic will be a focus of the studies in this thesis.

The bZIP transcriptional factors C/EBPα and C/EBPβ control cell differentiation and proliferation in multiple cell types and are suggested to be associated with eIF2~P (87, 88). Isoforms of C/EBPα and C/EBPβ can be produced by translation initiation at different AUGs of the encoded mRNAs, namely sites designated A, B1, B2, C and D (87). A short ORF (D) that is positioned out-of-frame between the A and B initiation codons regulates mRNA translation to produce multiple protein isoforms. The full-length protein isoforms of CEBPβ, designated LAP or LAP* are the result of translation initiation at the A, B1, or B2 sites (87). These LAP versions include an amino terminal transactivation domain, along with the carboxyl terminal bZIP domain, and act as transcriptional activators. By comparison the truncated protein isoform of CEBPβ, designated LIP, begins from the C start codon and is devoid of the activation region and functions as a transcriptional inhibitor (87, 89). The uORF D has an AUG in a weak Kozak sequence context, and optimization of this AUG results in increased translation from ORF D, allowing for a reinitation at the downstream start codon for ORF C, thereby enhancing LIP expression. eIF2~P is suggested to reduce translation from ORF D and the

subsequent translation of LIP, but the mechanisms involved in differential expression of the C/EBP isoforms are not well understood (88). This suggests that eIF2~P can not only regulate the levels of key regulatory proteins through preferential translation, but also isoform variants by differential recognition of start codons.

Along with the above mentioned transcripts, there are multiple genes that are suggested to be regulated translationally. Among these proteins are CDK inhibitor p27 (90), cyclin D1(91), G_1 cyclin CLN3(92), thrombopoietin (93), PDGF2 (94), BCL-2 (95), AdoMetDC (96), c-Myc (97), and SLC (98). The precise mechanisms controlling these key genes, and possible roles of eIF2~P, is only beginning to be fully appreciated. Clearly there is much to be learned about translational control and the ISR.

8. PERK functions in conjunction with additional stress sensors during ER stress

The ISR can function in conjunction with other stress-specific response pathways. For example, perturbation in the ER lumen due to accumulation of unfolded proteins can cause ER stress. This stress arrangement invokes a gene expression program called the Unfolded Protein Response (UPR), which leads to enhanced protein processing capacity of the secretory pathway and the ER-Associated Degradation (ERAD) pathway that facilitates ubiquitination and proteasome-mediated degradation of protein evicted from the ER to the cytosol (1). Cells sense ER stress by three transmembrane proteins: Inositol-requiring protein 1 (IRE1), ATF6, as well as the eIF2 kinase PERK (1, 3). During ER stress, eIF2~P by PERK reduces protein synthesis, decreasing the load of newly synthesized protein on the secretory pathway (1, 3, 99). Loss of PERK in cultured cells leads to high levels of protein synthesis, resulting in exacerbation of the ER stress

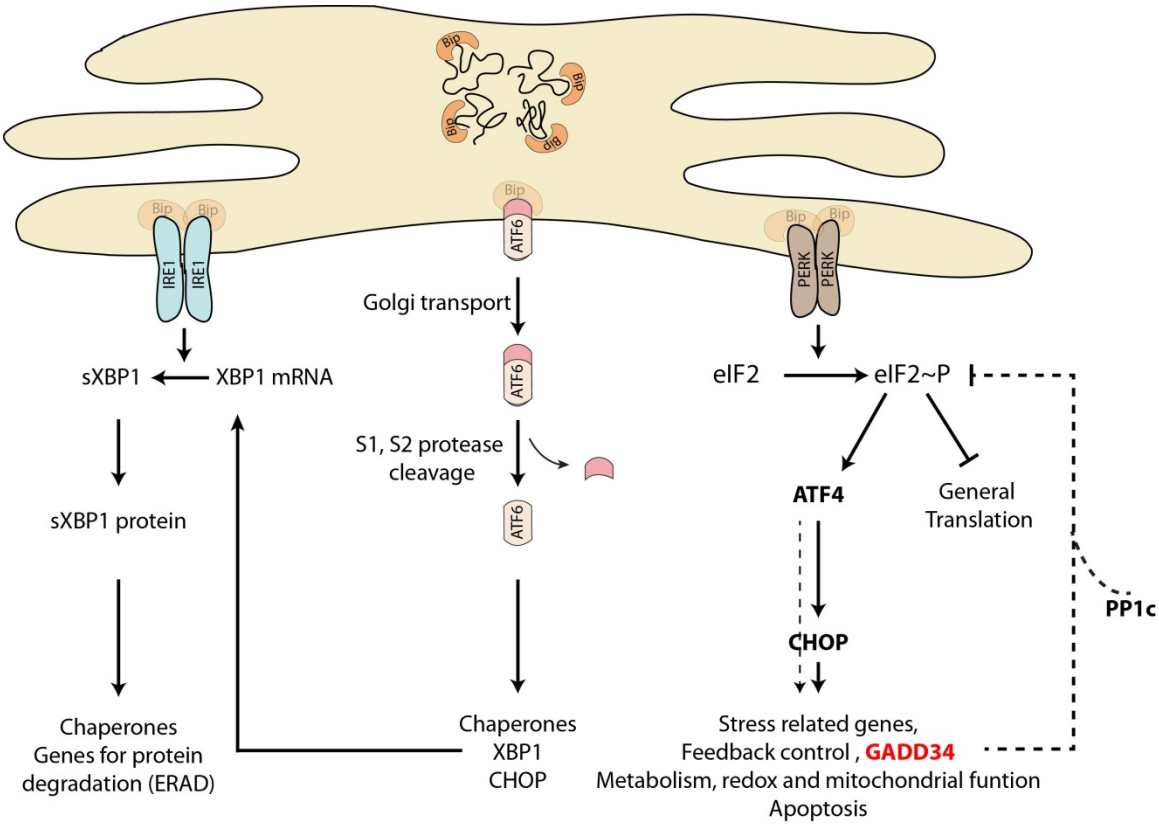


Figure 6. eIF2~P contributes to the Unfolded Protein Response that is activated in response to ER stress. The UPR is a program of gene expression designed to increase the ER capacity for protein folding and secretion. There are three arms of the UPR, which features the ER transmembrane proteins IRE1, ATF6, and PERK. In the presence of accumulated unfolded proteins in the ER, BiP (GRP78) dissociates from the IRE1 luminal portion and promotes oligomerization and auto-activation of IRE1. IRE1 endoribonuclease activity that facilitates the splicing of the *XBP1* mRNA to *sXBP1*, which encodes the active XBP1 transcription factor that induces genes encoding ER chaperones and factors involved in ER-Associated Protein Degradation (ERAD). During ER stress ATF6 translocates from ER to the Golgi, where S1 and S2 proteases cleave ATF6, releasing the ATF6 N-terminal portion into the cytosol. The cleaved ATF6 N-terminal portion acts as a transcription factor that induces genes that are important for

protein folding, and the XBP1 and CHOP transcription factors. Accumulation of unfolded proteins in the ER lumen is also thought to cause BiP dissociation from the luminal portion of the PERK. This allows PERK dimerization, followed by activation of cytosolic kinase domain through a process involving auto-phosphorylation. Activated PERK protein kinase phosphorylates the α subunit of eIF2 at serine 51. Phosphorylation of eIF2 elicits global translation repression, reducing the influx of newly synthesized proteins into the stressed ER. In parallel, eIF2 phosphorylation induces preferential translation of certain transcripts, such as *ATF4*. Transcriptional and translational program induced upon eIF2~P are called the Integrated Stress Response (ISR). The ISR functions as an integral part of the UPR during ER stress, but the ISR can also be activated by other stress conditions and function in conjunction with alternative stress signaling pathways. ATF4 targets genes involved in metabolism, anti-oxidation, mitochondrial functions, and the regulation of apoptosis. Furthermore, ATF4 directly increases the expression of additional transcription factors, such as CHOP and ATF3. Another target gene of ATF4 is *GADD34*, which recruits the protein phosphatase 1c to eIF2 dephosphorylation as part of a negative feedback mechanism. If the cells do not adapt to the stress conditions, then the induced UPR program shifts the cells from adaptation and survival to apoptosis.

and enhanced apoptosis (100). Furthermore, loss of *PERK*, and its downstream target *ATF4*, substantially ablate the UPR (10). Recently, it was reported that ATF4 is required for processing and activation of ATF6 during ER stress (101). This processing mechanism is further described below. Therefore, loss of the steps in the PERK/eIF2~P/ATF4 pathway not only diminishes expression of the ATF4-targeted genes, but also those activated by ATF6.

As mentioned earlier, many of the ATF4-targeted genes are required for resistance to oxidative stress, including heme oxygenase 1 (HMOX1) and sequestosome1/A170 (SQSM1) (10). The lumen of the ER has a high oxidizing environment, and it is a net consumer of glutathione (10, 102). Therefore, depletion of *ATF4* or *PERK* function renders cells sensitive to oxidative stress, which can be exacerbated during ER stress. Defects in import and metabolism of thiol-containing amino acids that are subject to regulation by ATF4 can also contribute to oxidative stress. Along with essential amino acids, growth and survival of $ATF4^{-/-}$ cells require supplementation with reducing substances, such as β-mercaptoethanol, N-acetyl cysteine, or glutathione (10).

The regulatory luminal domain of PERK shares homology with IRE1 (39, 103). This suggests that these two protein kinases share mechanistic features for activation in response to ER stress. IRE1 is a bi-functional enzyme, with protein kinase activity and sequence-specific endoribonuclease activity (104-106). The only protein that IRE1 is known to phosphorylate and regulate is itself (102, 105). Oligomerization-induced activation of IRE1 occurs through a mechanism that involves unfolded proteins by binding directly to the IRE1 luminal domain. Alternatively, the chaperone BiP was

reported to bind and repress IRE1 (39). However, with the accumulation of unfolded protein, BiP is suggested to be released from IRE1 and instead associate with the unfolded protein as a chaperone (108). This later mechanism would be similar to that described above for PERK. Activation of IRE1 involves autophosphorylation and conformational changes that induce IRE1 endoribonucleolytic activity (109). Enhanced endoribonucleolytic activity facilitates cytoplasmic splicing of mRNA encoding the X-box binding protein1 (XBP1) in mammals and homologue of the mammalian activating transcription factor/cAMP-response element (CRE)-binding protein (HAC1) in yeast (102, 106, 110, 111). Spliced *XBP1* mRNA leads to synthesis of an active bZIP transcription factor that induces a subset of the UPR genes involved in ER biogenesis and the ERAD-mode of protein clearance. sXBP1 enhances its own transcription, facilitating further amplification of *XBP1* synthesis, which is considered an autostimulatory loop. The UPR also induces the expression of genes required for phospholipid synthesis and membrane expansion to accommodate increased secretory demands (1). IRE1 is required for placenta development, and loss of IRE1 results in reduction of vascular endothelial growth factor-A and severe dysfunction of the labyrinth in the placenta (112). *IRE1*-deficient embryos that are supplied with normal placenta are developmentally viable (112). *XBP1* is also essential for B-cell differentiation (113, 114). Mutations in the autostimulatory loop that regulates *XBP-1* transcription were shown to be associated with bipolar mood disorders (115).

There are two ATF6 genes expressed in mammals,ATF6α and ATF6β, with the former being critical for activation of the UPR. During ER stress full-length ATF6 (90 kD) translocates from the ER to Golgi, where it is cleaved by site 1 and site 2 proteases,

designated S1P and S2P, respectively (116-118). The cytosolic portion of ATF6 (amino-terminal portion 50-kD in size) then translocates to the nucleus and activates UPR target promoters through ATF/CRE or ERSE binding elements. During this transcriptional activation, ATF6 is a bZIP protein that can form heterodimers with sXBP1 (110). Individual knockouts of the *ATF6* isoforms are not lethal in mice, but combined knockouts of *ATF6α* and *ATF6β* are early embryonic lethal, suggesting that ATF6 and the UPR are critical for development processes (119, 120).

As noted above, the PERK/eIF2~P/ATF4 pathway facilitates the activation of ATF6 possibly by enhancing the expression UPR genes that are required for processing and transport of ATF6 protein (101). Both ATF4 and XBP1 are also suggested to induce *ATF6* expression, and ATF6 in turn was reported to enhance *XBP1* transcript levels in response to ER stress (101, 110). Therefore, all three arms of the UPR are interconnected and contribute to induce a common set of genes required for proper protein secretion (102). ATF4 was also reported to contribute to expression of 4E-BP1 and REDD1 proteins, which are involved in mTORC1 regulation and function (121, 122). Therefore, eIF2~P is thought to be involved in cross-regulation between many different stress response pathways.

9. The role of eIF2~P in disease

As highlighted above, dysregulation of the eIF2 kinase pathways can be linked with many pathological conditions, such as Alzheimer disease, brain ischemia, diabetes and cancer. Wolcott-Rallison Syndrome (WRS) is a rare autosomal recessive disorder in humans due to loss of function mutations in *PERK* (12). WRS presents with neonatal

insulin-dependent diabetes and occurrence of epiphyseal dysplasia, osteoporosis and growth retardation. Knock out of *PERK* in mice exhibit similar phenotypes as in WRS (13, 14). Newborn *PERK*$^{-/-}$ mice have normal islets with insulin synthesis and secretion, but these pups develop diabetes during the first few weeks of life due to progressive loss of islet β cells. The exocrine pancreas in *PERK*-deficient mice is reduced for the synthesis of major digestive enzymes, leading to dysfunction and loss after the fourth postnatal week (13, 123). PERK is highly expressed in bone tissues and *PERK*$^{-/-}$ mice exhibit severe spinal curvature (hunchback), splayed hind limbs, and reduced locomotor activity, suggesting an important role for this eIF2 kinase in the skeletal system (3, 14). The underlying skeletal defects include deficient mineralization, osteoporosis, and abnormal compact bone development. *PERK*-deficient bone tissues have elevated procollagen levels, however corresponding type-1 collagen levels were reduced by two to four fold, suggesting a defect in procollagen processing or transport in the ER/Golgi secretory pathway (14). Studies on human cervical carcinomas suggested that PERK is required for tolerance to hypoxia and tumor growth. PERK is suggested to increase the intracellular antioxidants, thereby reducing oxidative DNA damage that can be triggered by reactive oxygen species (ROS) (85).

GCN2 knockout mice are viable, fertile, and exhibit no phenotypic abnormalities under normal growth conditions (124). However, *GCN2*$^{-/-}$ mice fed a leucine-deprived diet exhibit reduced skeletal muscle mass, and hepatic steatosis due to reduced lipid mobilization, resulting in some reduced viability (125, 126). Loss of *GCN2* was also shown to affect behavioral phenotypes and mnemonic processes. Genetic reduction of eIF2~P, either by depleting *GCN2* or by a heterozygous eIF2α serine 51 to alanine

mutation, lowered the threshold for late-long term potentiation (L-LTP) and reduced learning ability in several behavioral tasks (127, 128). Also, it was reported that mice lacking *ATF4* activity exhibit impaired late LTP (127-129). Cells present in the anterior piriform cortex (APC) can sense a diet deficient in indispensable amino acids (IAA) through GCN2. Wild type mice show aversion behavior to the nutrient-depleted diet, whereas *GCN2*$^{-/-}$ mice cannot (130, 131). Uncharged tRNA that accumulates in the APC was suggested to activate GCN2 in mice that were fed an amino acid depleted diet (130).

Although eIF2~P is required for adaptation to various stresses, constitutive activation of the eIF2 kinase pathway may lead to complications. Mutations in genes encoding eIF2B in humans can cause leukodystrophies, a fatal disorder characterized by vanishing white matter (VWM/CACH) (132-134). VWM is a chronic progressive disorder with progressive cerebellar ataxia, spasticity, inconstant optic atrophy and reduced mental abilities. Deterioration often follows acute fever or head trauma, i.e. periods of stress. This disease is found to be associated with missense mutations in each of the five eIF2B subunits, predominantly in subunits *eIF2B5* and *eIF2B2* (133, 134).

10. CHOP plays a critical role in eIF2~P-induced stress responses

CHOP is a bZIP transcription factor which can form homodimers, as well as heterodimers with other bZIP transcription factors, such as ATF4. *CHOP* was first found to be a transcript induced by genotoxic stress, and an alternative name for CHOP is growth arrest DNA damage 153 (GADD153) (135). Transcription of the *CHOP* gene is enhanced several fold by both ATF4 and ATF6 in response to ER stress (41, 136, 137). The transcriptional regulation of *CHOP* involves several regions of its promoter,

including ERSE, UPRSE, AARE, and CARE elements, which allow for *CHOP* transcriptional regulation (41). Finally, p38 MAP kinase regulates CHOP activity by direct phosphorylation of CHOP at serine-78, and this modification facilitates the nuclear translocation of CHOP for enhanced transcription of its target genes (138).

As noted above, both ATF4 and CHOP directly induce the transcription of *GADD34*, which facilitates dephosphorylation of eIF2 and feedback control of the stress response pathway (34, 35). This allows UPR gene transcripts to be translated more efficiently in response to ER stress. CHOP antagonizes ATF4 activity in *ASNS* transcriptional regulation, by enhancing expression of ATF3, which then displace ATF4 at later hours of arsenite stress (139, 140). Induction of *TRB3* is also a CHOP-dependent process during nutrient deprivation (141, 142). TRB3 can bind to ATF4 and antagonize its transcriptional activity (143). Thus, CHOP can contribute to feedback regulation of the ISR by multiple mechanisms.

CHOP is associated with a range of pathologies triggered by ER stress (2). Certain mouse models of diabetes, such as the Akita mouse, result from insulin gene mutations that affect insulin folding process (144). As a consequence, the unfolded insulin forms aggregates in a dominant fashion, rendering insulin non-functional (145, 146). The aggregated mutant insulin proteins in the islet β cells can trigger ER stress, inducing cell death and depletion of the β cell mass. Deletion of which in Akita mice significantly alleviates depletion of the β cell mass, supporting the idea that CHOP can play a major role in triggering cell death upon chronic ER stress (146). Further supporting this model, loss of *CHOP* reduces apoptosis in cultured cells treated with pharmacological agents inducing ER stress, such as A23187 or tunicamycin (147).

Enhanced renal dysfunction is also correlated with increased *CHOP* expression in mice injected with tunicamycin, compared to *CHOP*$^{-/-}$ mice (34). Charcot-Marie-Tooth 1B neuropathy in humans exhibits demyelination condition, in which the neuronal cells have the P0 glycoprotein with serine 63 deletion (P0S63del) (148). P0S63del transgenic mice produce similar neuropathies. Ablation of CHOP induction in these mice restores motor function and reduces demyelination (149). Finally, deletion of *CHOP* promotes survival of dopaminergic neurons from the toxic effects in a model of Parkinson's disease (150). Together, these studies indicate that CHOP is an important participant in the balance between cell survival and death in response to ER stress, and other stress conditions.

11. Role of CHOP in apoptosis induced by ER stress

CHOP is suggested to contribute to enhanced apoptosis by several mechanisms. For example, CHOP together with the LIP isoform of C/EBPβ was reported to bind to the *BCL2* promoter, repressing the transcription of the pro-survival factor (151). ER stress induction of CHOP also enhances *BIM* transcription. CHOP forms heterodimers with C/EBPα and directly binds to the *BIM* promoter region (TGCAAT) that is present on the first intron (152). The proapoptotic function of *BIM* can be further regulated by post-translational control mechanisms. Protein phosphatase 2A dephophorylates the BIM protein in response to ER stress, preventing the subsequent ubiquitination and degradation of BIM, thereby increasing BIM protein levels (152). Decreased levels of BCL2 and increased levels of BIM promote BAX/BAK oligomerization at the mitochondrial membrane, which facilitates caspase activation and promotes apoptosis

(153). In human HCT116 cancer cells, CHOP increases the levels of death receptor 5 (DR-5) during ER stress. TRAIL-mediated activation of DR-5 promotes intrinsic apoptosis, through activation of the caspase-8/BID/BAX pathway (154).

CHOP increases the expression of ER oxidase 1 alpha (*ERO1α*), which is important for formation of disulfide bonds and can create oxidative stress that elicits a cascade of events that trigger apoptosis (34). Furthermore, ERO1α activates ER-resident calcium release channel protein, IP3R. Hyperoxidizing of the environment of the ER lumen dissociates the repressive interaction between ERp44 (disulphide isomerase) and IP3R, leading to activation of the calcium channel (155). Increases in cytosolic calcium levels can further activate CaMKII. Downstream effectors of CaMKII include apoptosis-inducing factors, such as NADPH oxidase subunit Nox2, JNK, Fas and STAT1 (153). While the initial induction of CaMKII is ERO1α independent, the ERO1α sustains the CaMKII activity (155). CHOP also transcriptionally enhances *TRB3*, which can block the pro-survival effect of AKT (141). Finally, CHOP induction of *GADD34* during prolonged ER stress results in premature resumption of protein synthesis, which can lead to further accumulation of unfolded proteins, ultimately leading to proteotoxicity and cell death (34). How these myriad of transcription events switch cells from adaptation and survival to apoptosis in response to prolonged stress conditions is still not fully understood. This thesis will focus on the key regulatory events contributing to enhanced *CHOP* expression during ER stress, and the role elevated CHOP levels can contribute to apoptosis.

EXPERIMENTAL PROCEDURES

1. Plasmid constructions

RT-PCR was performed with total RNA isolated from mouse embryonic fibroblasts (MEF). Prepared cDNAs were used to PCR amplify the HindIII-Nco1 fragment DNA encoding the 5'- leader of the *CHOP* mRNA, which was inserted between HindIII and NcoI restriction sites in a pGL3-derived plasmid (79). Primer sequences based on the *CHOP* mRNA leader obtained from the RIKEN (The Institute of Physical and Chemical Research, Japan) database are as follows: sense 5'-GCTCAAGCTTCTAACACGTCGATTAT-3', antisense 5'-TCATGAGTGCCATGACTGCACGTGG-3' (GeneBank accession number BC013718). The resulting P_{TK}-*CHOP-Luc* plasmid contains the *CHOP* 5' leader fused to a luciferase reporter downstream of a constitutive TK promoter. The uAUG1 and uAUG2 individually or in combination were mutated to AGG by using a site directed mutagenesis kit (Stratagene) following the manufacturer's instructions (Stratagene). The stop codon UGA in the uORF of P_{TK}-*CHOP-Luc* was mutated to GGA in combination with the uAUG mutations, as indicated. Codon 24 (AGA) in uORF1 of P_{TK}-*CHOP-Luc* was mutated to UGA to generate the luciferase reporter with short uORF encoding amino acids 1-23. All plasmids were sequenced to ensure that there were only the desired changes. A PstI site was created 10 nucleotides upstream of the uORF in P_{TK}-*CHOP-Luc* plasmid to insert a stem loop structure or 120 nucleotide sequence. The stem-loop structure 5'-CTGCAGCCACCACGGCCCCCAAGCTTGGGCCGTGGTGGCTGCAG-3' was derived from previously published report (79). Extension of the 5'-leader was achieved by inserting a 120-nucleotide sequence that is devoid of any start and stop

codons and strong predicted secondary structures into the PstI site. An uORF with a strong Kozak initiation context was generated by replacing TATATC***ATG***TTGAAG***ATG***A sequence in the CHOP uORF with the Kozak consensus sequence GCCACC***ATG***G (initiation codons in bold) by using the sequence and ligation–independent cloning (SLIC) method (156). In brief, the DNA fragment was amplified using Pfu Turbo DNA polymerase (Stratagene) with P_{TK}-*CHOP-Luc* as template strand and the following primers: sense 5'-GCCACCATGGCGTATAGCGGGTGGCAGCGACAGAGCCAGAATAAC-3', antisense 5'-ATACGCCATGGTGGCGATATAATCGACGTGTTAGAAGCTTATGCA-3'. The template plasmid DNA was digested with DpnI and amplified fragment DNA was purified using a PCR purification kit (Qiagen). 1 µg of the prepared DNA was treated with 0.5 units of T4 DNA polymerase (New England Biolab) in a 20 µl reaction volume containing 1X NEB buffer 2 and BSA. Following incubation at room temperature for 20 minutes, the reaction was terminated by the addition of 2.2 µl of 10 mM dCTP nucleotides. The reaction mixture was then incubated with 1X ligation buffer (NEB) and 20 ng of RecA protein (Epicenter Biotechnologies) at 37^{o}C for 30 min. A portion of the above annealed reaction mixture was used for bacterial transformation. 5'-phophorylated primers (IDT) were used to construct uORF-Luc fusion plasmid in which *CHOP* uORF was directly fused to the luciferase coding sequence. P_{TK}-*CHOP-Luc* was used as the template strand and the 5'-phosphorylated primers used were as follows: WT (34aa) uORF-Luc antisense 5'-GGTGTGGTGGTGTATGAAGATGCAC-3', Δ 24-34 amino acids uORF-Luc antisense 5'-GGAACACTCTCTCCTCAGGTTCCG-3', Δ 14-34 amino acids uORF-Luc antisense 5'-/5ATTCTGGCTCTGTCGCTGCCACCCGCT-3', uORF-

Luc sense 5'-GAAGACGCCAAAAACATAAAGAAAGGCC-3'. WT (34aa) uORF-Luc construct was used as template strand to create the Δ14-23 amino acids uORF-Luc plasmid that encodes for uORF peptide deleted for amino acids 14-23 fused to the luciferase by using the following primers: sense 5'-AGAAGGAAGTGCATCTTCATACACCACCACAC-3', Δ14-23 aa uORF-Luc antisense 5'-ATTCTGGCTCTGTCGCTGCCACCCGCT-3'. The template plasmid DNA was digested with DpnI and amplified fragment DNA was purified using a PCR purification kit (Qiagen). 1 µg of amplified fragment DNA was used for ligation using T4 DNA ligase (Invitrogen) and the ligated DNA was transformed to BL-21 bacterial strains. The eIF1 encoding plasmid DNA was a gift from Dr. Ivanov (University of Utah) (157).

2. Cell culture and dual luciferase assays

MEF cells that were derived from *S/S* (wild-type eIF2α) and *A/A* (mutant eIF2α-Ser51A) mice were previously described (158, 159). MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM from Cellgrow) supplied with 10% FBS, 1 mM non-essential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin. ER stress was induced in MEF cells by the addition of either 0.1 µM or 1 µM thapsigargin to the medium, followed by incubation for up to 12 hrs, as indicated. Plasmid transfections were performed using the *S/S* and *A/A* MEF cells grown to 40% confluency and with the FuGENE 6 transfection reagent (Roche Applied Science). Co-transfections were carried out in triplicates using wild type or mutant versions of the *CHOP-Luc* fusion plasmids and a *Renilla* luciferase plasmid serving as an internal control (Promega). 24 h after transfection, MEF cells were treated with 0.1 µM

thapsigargin for 12 h, or with no ER stress. Dual luciferase assays were carried out as described by the Promega instruction manual. Values are a measure of a ratio of *Firefly* versus *Renilla* luciferase units (relative light units) and represent the mean values of three independent transfections. Results are presented as means ± S.E. that were derived from three independent experiments. Parallel to the dual luciferase assays the amount of *Firefly* luciferase mRNA in each transfected condition was measured by using qRT-PCR method. P_{TK}-*CHOP-Luc* reporter plasmid co-transfected with pcDNA or plasmid DNA encoding eIF1 into MEF cells, and then cultured for 36 hours. Stress was induced with 0.1 uM Tg for 6 hrs, and then cell lysates were collected for dual luciferase assays. *Renilla* luciferase plasmid was included with co-transfections to serve as internal control. Luciferase assays were performed according to manufacturer instructions (Promega).

3. Preparation of protein lysates and immunoblot analyses

MEF cells cultured for indicated stress conditions or no stress were washed twice with cold phosphate buffered saline (pH 7.4) followed by lysis with a solution containing 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 100 mM NaF, 17.5 mM β-glycerolphosphate, 10% glycerol supplemented with protease inhibitors (100 µM of phenylmethylsulfonyl fluoride, 0.15 µM aprotinin, 1 µM leupeptin, and 1µM of pepstatin). Lysates were subjected to sonication for 30 seconds and precleared by centrifugation. Protein content was determined by using a Bio-Rad protein quantitation kit according to the manufacturer instructions. Equal amounts of proteins were separated by SDS-PAGE and proteins were then transferred to nitrocellulose filters. Molecular weight markers were included for size determination of proteins in the immunoblot

analyses. Transferred filters were then incubated in TBS-T solution containing 20 mM Tris-HCl (pH 7.9), 150 mM NaCl, and 0.2% Tween 20 supplemented with 4% nonfat milk, followed by incubation with TBS-T solution with primary antibody specific to the indicated protein.

ATF4 antibody was prepared against recombinant protein (66). CHOP (sc-7351) antibody was obtained from Santa Cruz Biotechnology, and β-actin monoclonal antibody (A5441) was purchased from Sigma. Polyclonal antibody that specifically recognizes phosphorylated eIF2α at Ser-51 was purchased from BioSource (44–728G). Monoclonal antibody that recognizes either the phosphorylated or non-phosphorylated forms of eIF2α was provided by Dr. Scot Kimball (Pennsylvania State University, College of Medicine, Hershey, PA). Cell lysates from MEF cells transfected with the indicated plasmids were blotted for Firefirefly luciferase protein by using antibody purchased from Promega (G7451). Filters were then washed three times in TBS-T followed by incubation with horseradish peroxidase-labeled secondary antibody and chemiluminescent substrate. Proteins in the immunoblot were visualized by exposing filters to x-ray film or by imaging using the LI-COR Odyssey system. Images shown in the figures are representative of three independent experiments. Cells transfected with plasmid DNA encoding eIF1 or pcDNA were treated with 0.5 μM thapsigargin for the indicated time points, and then protein lysates were collected for protein analysis. Antibody that recognizes eIF1 was obtained from Abnova (M01A).

4. Determining the transcriptional start site of *CHOP* mRNA

The cDNAs corresponding to the 5'-ends of the *CHOP* mRNAs expressed in S/S MEF cells treated with 0.1 μM thapsigargin, or no stress, were amplified by using a RNA ligase-mediated RACE kit (RLM-RACE kit, Ambion) according to the manufacturer's instructions. Total RNA was first isolated using an RNeasy mini kit (Qiagen). Briefly, 10 μg of total RNA was treated with calf intestinal phosphatase to remove 5'-phosphates from RNAs other than mRNAs with intact 5'-cap structures. Later the 5'-cap structures were removed by using tobacco acid pyrophosphatase, leaving 5' monophosphates that were ligated using T4 RNA ligase to a 45-base pair RNA adapter oligonucleotide supplied in the RACE kit. The RNA preparations were then subjected to random primed reverse transcription (RT) reaction and nested PCR to amplify the 5'-end of endogenous *CHOP* transcripts, as well as transfected thymidine kinase-minimal promoter driven *CHOP-Luc* mRNAs. The sense primers corresponding to 5'-RACE adapter sequences were provided by manufacturer. Anti-sense primers corresponding to the endogenous *CHOP* mRNA were as follows: outer primer 5'-GGACGCAGGGTCAAGAGTAG-3', inner primer 5'-TCATGAGTGCCATGACTGCACGTGG-3'. The outer primer used for amplifying *CHOP-Luc* mRNA 5'-end was 5'-CGAATTCGAACACGCAGAT-3', which was combined with the same inner primer listed above. The amplified product was then analyzed using electrophoresis and a 1.2% agarose gel. The prominent DNA bands were excised, gel purified, and sequenced. The transcriptional start site was determined as the first nucleotide that is 3' to the adapter sequence ligated to the 5'-end of the mRNAs.

5. RNA isolation and real time PCR

MEF cells transfected with the indicated plasmids and designated stress conditions were harvested, and total cellular RNA was prepared using TRIzol reagent (Invitrogen) according to the instruction manual. Contaminating DNA was digested with RNase free DNase (Promega). Single-strand cDNA synthesis was carried out using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Quantitation of relative mRNA levels was performed using the SYBR green PCR mix (Applied Biosystems) and Roche Light Cycler 480 PCR system. The amount of firefly luciferase mRNA was measured using *Renilla* luciferase as an internal control. The oligonucleotide primers used were as follows: Firefly luciferase 5'-CTCACTGAGACTACATCAGC-3' and 5'-TCCAGATCCACAACCTTCGC-3', *Renilla* luciferase 5'-GGAATTATAATGCTTATCTACGTGC -3' and 5'-CTTGCGAAAAATGAAGACCTTTTAC-3'. The endogenous *CHOP*, *ATF4* and β-actin mRNA levels were measured using the following oligonucleotide primers: *CHOP* 5'-CCTAGCTTGGCTGACAGAGG-3' and 5'-CTGCTCCTTCTCCTTCATGC-3', *ATF4* 5'-GCCGGTTTAAGTTGTGTGCT-3' and 5'-CTGGATTCGAGGAATGTGCT-3', β-actin 5'-GTATGGAATCCTGTGGCATC-3' and 5'-AAGCACTTGCGGTGCACGAT-3'. Quantitation of target genes was normalized using the reference 18S rRNA to compensate for inter-PCR variations. The 18S rRNA primers used in the PCR assays were 5'- CGGCTACCACATCCAAGGAAGG-3' and 5'-CCCGCTCCCAAGATCCAACTAC-3'. The endogenous *CHOP*, *GADD34*, *BCL2*, and *BIM* levels in WT-uORF-CHOP and ΔuORF-CHOP cells were measured using Applied Biosystems TaqMan gene expression assays. Light Cycler 480 software (Version

1.2.9.11) was used to perform quantification and to determine the crossing point (Cp) values. Values are a representation of three independent experiments, with standard deviations as indicated.

6. Polysome analysis of *CHOP* translational control

MEF cells were cultured in Dulbecco's modified Eagle's medium as described and were treated with 1 μM thapsigargin for 6 hours or no stress. Prior to harvesting, cells were treated with 50 μg/ml cycloheximide and incubated at 37^{o}C for 10 minutes. Cells were washed with cold phosphate buffered saline (pH 7.4) solution containing 50 μg/ml cycloheximide and cell lysates were prepared in a solution containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, and 0.4% Nonidet P-40 supplemented with 50 μg/ml cycloheximide. Cell lysates were passed through a 23-gauge needle and incubated on ice for 10 minutes. The cell lysate was precleared with brief centrifugation (10,000 rpm for 10 minutes at 4^{o}C) then layered onto a 10-50% sucrose gradient solution containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, and 50 μg/ml cycloheximide. The sucrose gradients were then subjected to centrifugation in a Beckman SW-41Ti rotor for 2 h at 40,000 rpm at 4^{o}C. A portion of un-fractionated cell lysate was used for determining total mRNA levels of *CHOP*, *ATF4* and β-actin. Gradients were fractionated using a Biocomp Gradient Station, and absorbance of RNA at 254 nm was recorded using an in-line UV monitor. Equivalent amounts of synthetic Poly(A)$^{+}$ luciferase RNA (10 ng/ml) purchased from Promega were added to each collected fraction. RNA was isolated from each fraction using TRIzol LS reagent (Invitrogen) and single-stranded cDNA synthesis was performed using Superscript III reverse

transcriptase (Invitrogen) according to manufacturer's instructions. Prepared cDNA was used to measure the relative mRNA levels of *CHOP*, *ATF4* and β-actin. qRT-PCR data were normalized with the luciferase mRNA that was added as a spike-in control prior to RNA isolation. The results generated in this study included three independent experiments, with standard deviations as indicated.

MEF cells cultured to 40% confluency were also transfected with P_{TK}-*CHOP-Luc* plasmid or mutant derivatives Δ*ATG1*, Δ*ATG2*, and Δ*TGA* in the 5'-leader using Fugene6 (Roche) transfection reagent. After 24 hours of transfection cells were treated with 0.1 μM thapsigargin for up to 6 hours or no stress. Cell lysates prepared were then subjected to sucrose gradient analyses and fractionated, as described above. Equivalent amounts of bacterial spike-in control RNA (Affymetrix) was added to each sucrose fraction to serve as internal control in RNA isolation and in qRT-PCR analysis. The use of bacterial spike-in RNA was included in this study as the luciferase reporter mRNA were the focus of the translational control measurements. RNA isolated from each fraction was used in preparation of single-stranded cDNA synthesis as described. The amount of firefly luciferase in each fraction was quantitated and normalized to *THR* mRNA included in bacterial spike-in RNA mix. Oligonucleotide primers used for *THR* mRNA measurement were as follows: forward 5'-AGGATGACGAGACCCAAATG-3' and reverse 5'-TGATCGCAGCAATGAGGATA-3'.

7. Preparation of a *CHOP*$^{-/-}$/FRT recipient cell line

MEF cells that were derived from *CHOP*$^{-/-}$ mice were previously described (147, 159). These cells were grown to 25% confluence in a 60 mm dish containing DMEM

media (Cell Grow) supplied with 10% FBS, 1 mM non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. These cells were transfected with 1 μg of linearized (SacI) FRT/Lac-Zeo plasmid construct (Invitrogen) using Fugene reagent (Roche) and allowed to grow for 2 days under 5% CO_2 condition in 37^{o} C incubator. Following the transfection, cells were split into 10 cm dishes at low cell density, and allowed to attach the bottom of the plate for 4 hours. The media was then replaced with fresh media containing 175 μg/ml zeomycin every 2 days. The cells were selected for zeomycin resistance and screened for the presence of the FRT site using Southern blotting procedure. Genomic DNA was isolated from Zeomycin resistant *CHOP*$^{-/-}$ cells using the Genomic DNA isolation kit (Promega). Approximately 20 μg of genomic DNA was subjected to HindIII restriction digestion in an overnight incubation, and then the digested fragments were separated using by electrophoresis in an agarose gel. The separated DNA fragments were transferred onto Nylon N+ membranes using capillary electrophoresis (160). A *LacZ* gene DNA fragment (~1-kb), which was obtained from FRT/Lac-Zeo plasmid restriction digestion, was used as a template for probe preparation. Radioactive ^{32}P-dCTP nucleotides (Perkin Elmer) were used to label the probe following the manufacturer's instructions (Readprime-II-GE Healthcare). Quickhyb hybridization buffer was used in the hybridization procedure following manufacturer's instructions (Quickhyb-Stratagene). The unbound radioactive probe was washed thoroughly with low strength Quickhyb buffer and then the blot was analyzed using a Phosphor imager system. The *CHOP*$^{-/-}$ cells harboring single FRT site were selected and frozen for storage and further analysis. Furthermore, to confirm the FRT/Lac-Zeo integration into the

genome of *CHOP*$^{-/-}$ cells, β-galactosidase assays were performed following instructions (Invitrogen).

8. Construction of the WT-uORF-CHOP/FRT or ΔuORF-CHOP/FRT reporters

Mouse genomic DNA was used to amplify 1-kb of the *CHOP* promoter region using the following primers that included flanking restriction sites BglII and HindIII (underlined sequences): sense primer 5'-CTGAGATCTCTGTGTTTCCTCTGATGACCCAGT-3' and antisense primer 5'-GTTAGAAGCTTGCTCAAGATAACTGACCTCAAGA-3'. The CMV promoter region in the pcDNA/FRT plasmid was replaced with above 1-kb *CHOP* promoter DNA fragment between the BglII and HindIII restriction sites, generating plasmid pcDNA/CHOP/FRT. Mouse cDNA that prepared from total RNA was used to amplify the DNA fragment that encode CHOP full length ORF and 5'-leader using the following primers that included flanking restriction sites HindIII and XbaI (underlined sequences): sense: 5'-GCTCAAGCTTCTAACACGTCGATTAT-3', and antisense 5'-CGCTCTAGATGTTCATGCTTGGTGCAGGCTGAC-3'. The amplified *CHOP* DNA was inserted between the HindIII and XbaI sites in plasmid pcDNA/CHOP/FRT, generating plasmid pWT-uORF-CHOP/FRT. Plasmid WT-uORF-CHOP/FRT was then used as a template to delete the uORF function (ΔuORF) present in 5'-leader of *CHOP* by mutating ATG1 and ATG2 each to AGGs using site directed mutation strategy, as described above. This resulted in a mutant plasmid ΔuORF-CHOP/FRT that was devoid of the uORF of *CHOP*. The plasmids WT-uORF-CHOP/FRT and ΔuORF-CHOP/FRT

were used to generate stable cell lines that express *CHOP* mRNA transcripts containing 5'-leaders with either the WT uORF or ΔuORF.

9. Stable expression of *CHOP* in FRT cells

FRT recipient *CHOP*$^{-/-}$ cells were grown to 25% confluence in a 60 mm dish containing DMEM media (Cellgrow) that was supplied with 10% FBS, 1mM non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. These cells were co-transfected with pOG44 plasmid that encodes flippase enzyme, along with above WT-uORF-CHOP/FRT or ΔuORF-CHOP/FRT plasmids, according to the manufacturer instructions (Invitrogen). After two days of transfection, the cells then were split at low densities into 10 cm dishes, and incubated for 4 hour at 37^{o}C and 5% CO_2 to allow the cells to attach to the culture dishes. The media was then supplemented with hygromycin to 200 μg/ml concentration, and cells were selected for hygromycin resistance for further analysis for expression of *CHOP* mRNA and protein.

10. Cell survival assays

CHOP$^{-/-}$ cells and FRT recipient cell lines containing *WT uORF-CHOP* or Δ*uORF-CHOP* stably integrated into their genomes were analyzed for resistance to the indicated stress agents using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega G4001). This colormetric assay is based on the ability of living cells to convert a tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in the dye solution into a colored formazan product. This assay was performed in a 96 well plate format and measured using a plate reader at an absorbance of 570 nm. 5,000 cells

were plated per well in a 0.1 ml volume of DMEM supplemented with 10% FBS, , 1 mM non-essential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin. After overnight incubation, 25 nM thapsigargin, 1 µM MG132, or 0.5 µM tunicamycin, was applied to each well. Cells were incubated in the presence of the stress agent for 1, 3, 6, 12, or 18 hours, or no treatment. At the indicated times, the media containing the stress agent was removed by gentle aspiration with 0.5-10 µl pipet tips, and cells were then washed with warm media prior to incubation in DMEM in the absence of stress for a total of 24 hours from the beginning time of the stress treatment. For example, the cells treated with thapsigargin for 1 hour, were washed and then cultured in the absence of this stress agent for 23 hours. After the 24 hour period, 15 µl of the dye solution was added to a final volume of 115 µl, and then incubated with the cells for 4 hours, followed by the addition of 100 µl solubilization solution, as described by the manufacturer's protocol. After the formazan crystals were solubilized, the absorbance of the solution was measured at 570 nm using a Spectra Max 340 96-well plate reader (Molecular Devices).

11. Polysomal RNA preparation for micro array analysis

MEF cells were cultured in Dulbecco's modified Eagle's medium as described above, and treated with 5 µM thapsigargin for 6 hours or no stress. Prior to harvesting, cells were treated with 50 µg/ml cycloheximide and incubated at 37^{o}C for 10 minutes. Cell lysates were subjected to sucrose gradient centrifugation and the polysomal fractionation was performed as described in *CHOP-Luc* polysomal analysis procedure. A total of 14 fractions were collected from the top of the gradients into cold microfuge tubes and immediately placed on ice. Each fraction was adjusted to 0.5% SDS, and the 14

fractions were combined to form three pools as follows: fractions 1–4, 5–8, 9–14 were combined as pools 1, 2, and 3, respectively. In parallel, total RNA was isolated from unfractionated lysates for transcriptional analysis. To each 1 ml fraction pool, equivalent amounts of synthetic Poly(A) luciferase RNA (10 ng/ml), along with bacterial spike-in control RNA, were added. Synthetic luciferase RNA served as a control for the efficiency of RNA isolation. The bacterial spike-in RNA was purchased from Affymetrix and has different concentrations of each of the four exogenous, premixed, polyadenylated prokaryotic RNA controls. The prokaryotic genes used as spike-in controls have limited cross-hybridization with mammalian sequences but have target sequences on the Affymetrix arrays; hence the spiked in bacterial RNA serve as controls for both mRNA isolation and hybridization efficiency. RNA was precipitated at -70^{o}C with 2.5 volumes 100% ethanol and purified using QIAGEN RNeasy midi-columns. The quality of RNA was determined using an Agilent Bioanalyzer and quantitated by absorbance at 260 nm. For total unfractionated RNA, samples were subjected to ethanol precipitation. Total RNA was isolated, analyzed, and stored the same way as the RNA from polysomal fractions.

12. Microarray hybridization and normalization using spike-in controls

The RNA was then labeled using the standard Affymetrix protocol for 3′-IVT arrays (Affymetrix, Santa Clara, CA). Labeled cRNA was hybridized for 17 hours to the Affymetrix Mouse Genome 430 2.0 Array. The signal values and detection calls were derived using the MAS5 algorithm in Affymetrix GeneChip Operating Software. Affymetrix arrays were hybridized and scanned at the Center for Medical Genomics,

IUSM following standard protocols. Normalization across arrays was disabled in the MAS5 algorithm. The spike-in control RNA probe-set raw intensity values spanned the range of gene expression from low to strongly expressed genes. These values were used to normalize gene expression values across arrays as previously described (161). First, the spike-in control probeset expression values were log(2) transformed. An average probeset value for each array was calculated by taking the mean log(2) intensity of the 3'- and middle probes for the all of the control RNAs. This value was used to generate a normalization factor for each array, termed the Mod factor. The Mod factor is calculated by 10 – Spike-in probeset average value on a log(2) transformed scale. The gene expression values were normalized by adding this constant to all log(2) transformed expression data for that sample, thereby standardizing expression across all the arrays. Probe sets were eliminated from further analysis if an absent call was determined in greater than 33.3% of both the control and treated unfractionated samples. The same probe sets were eliminated from the fractioned polysomal RNA samples, and rest of the probe sets were considered for further analysis.

13. Genome-wide analysis of mRNA translation control in response to ER stress

Differentially translated genes were identified using the data generated from the three pools following a modification of the procedure employed for the unfractionated RNA analysis. This analysis is based upon the fact that the majority of mRNA bound to multiple ribosomes are in pool 3, while pools 1 and 2 contain mRNAs bound to no ribosomes and, at most, 1–3 ribosomes, respectively. Consequently, the percentage of a transcript that resides in pool 3 is a measure of translational efficiency. For each replicate

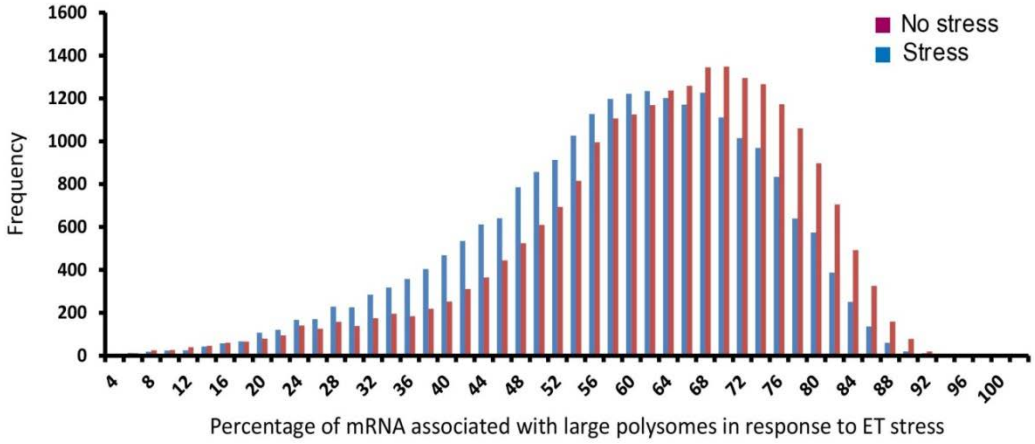
control and treated sample, we calculated the fraction of normalized log(2) transformed mRNA intensity in pool 3 divided by the total mRNA intensity (pool 3 / [pool 1+2+3]). Statistical analysis on the biological replicates was performed using student t-test to derive p-values for each probe set.

RESULTS

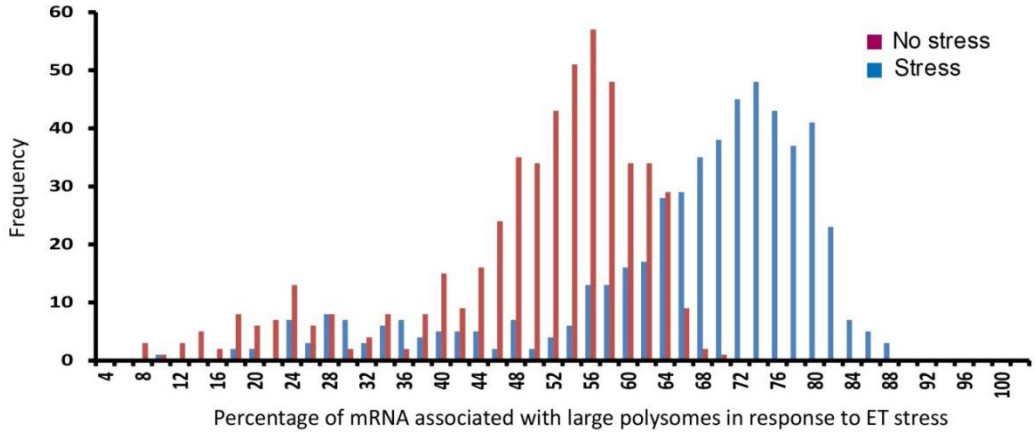
1. Analysis of genome-wide mRNA association with polysomes in response to ER stress

ER stress induces global translation repression, allowing cells to reduce the amount of newly synthesized protein entering the secretory pathway, while it reprograms gene expression to alleviate stress-induced damage. During this global translation repression, selected mRNAs such as *ATF4* and *ATF5* are preferentially translated. We propose that there may be additional transcripts that are preferentially translated during ER stress and these would be central to the efficacy of the eIF2~P induced ISR. To identify mRNAs with enhanced translation efficiencies during ER stress we performed a microarray analysis of transcripts fractionated by sucrose gradient centrifugation. To obtain sustained levels of eIF2~P, MEF cells were treated with 5 μM thapsigargin for 6 hours, or to no stress. ER stress for 6 hours will elicit the ISR transcriptome, which would be present for analysis of preferential translation. Polysomes were separated by centrifugation of cytoplasmic extracts in 10 to 50% sucrose density gradients, followed by fractionation. Polyadenylated prokaryotic mRNA was added to each collected fraction to serve as control for purification and array hybridization. The mRNA migrated with large polysomes are considered to be highly translated. The collected fractions were grouped into 3 pools: Pool 1 constitutes mRNAs that are bound to mRNP particles or 40S ribosomal subunits. Pool 2 represents mRNAs bound to one to three ribosomes. Transcripts in pool 2 are thought to be inefficiently translated or are very short in length. Pool 3 consists of mRNAs bound to four or more ribosomes, which are suggested to be

A)


No stress
Stress
Frequency
1600
1400
1200
1000
800
600
400
200
0
4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84 88 92 96 100
Percentage of mRNA associated with large polysomes in response to ET stress

B)


No stress
Stress
Frequency
60
50
40
30
20
10
0
4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84 88 92 96 100
Percentage of mRNA associated with large polysomes in response to ET stress

C)

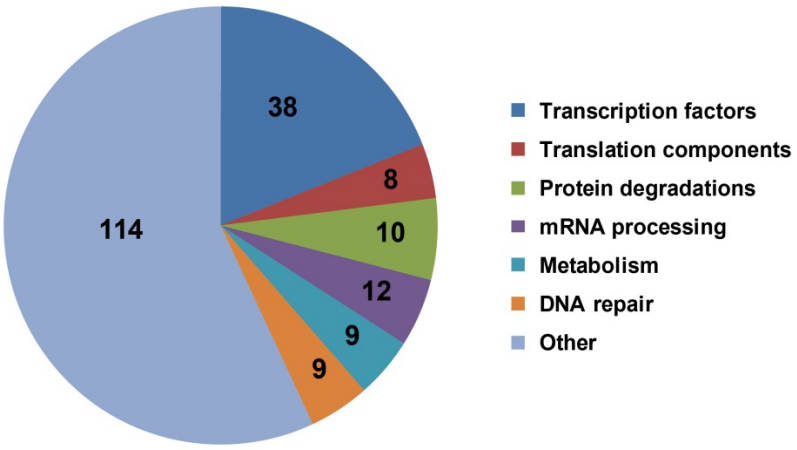

38
8
10
12
9
9
114
Transcription factors
Translation components
Protein degradations
mRNA processing
Metabolism
DNA repair
Other

Figure 7. Distribution of gene transcripts among polysomes in response to ER stress. MEF cells were treated with thapsigargin, or no stress, lysed, and the cell preparations were layered onto 10 to 50% sucrose gradients. The sucrose gradients were then subjected to centrifugation, and fractionation using a Biocomp gradient fractionator. Equal amounts of bacterial RNA control were spiked into obtained fractions as a control for cDNA synthesis and mRNA quantitation. RNA was isolated from the resulting fractions, and then subjected to microarray hybridization. Signal intensities from each array were normalized to spike-in control. The percentage of each gene transcript associated with large polysomes was calculated from the obtained signal intensities corresponding to polysomal mRNA. (A) and (B) The number of gene transcripts was plotted for each of the percentage of polysome association from cells subjected to ER stress (Blue), or to no stress (Red). Panel A represents polysomal association of genes from the genome wide analysis, and panel B represents those genes whose polysome association was enhanced by 10% or greater ($p<0.05$) in response to ER stress. (C) The top 200 genes that showed greater association with large polysomes in response to ER stress compared to control condition were selected from the list. These genes were categorized based on their molecular function that is represented by their gene ontology terms (GO-terms) from the microarray data.

the well-translated transcripts. mRNAs purified from the three pools were subjected to oligonucleotide Affymetrix microarray (mouse 430.2 arrays) hybridization.

ER stress affects the distribution of mRNAs between large polysomes to small polysomes or mRNP particles and thus alter the efficiency of protein synthesis. Hence, the proportion of mRNAs associated with large polysomes can be used as measure of mRNA translation. In this approach the percentage of a given mRNA associated with pool 3 was used as an index of mRNA translation efficiency. Changes in mRNA translation efficiencies in response to ER stress compared to no treatment condition were observed (Fig. 7A and B). Translation efficiencies for ~22,500 probe sets were obtained after filtering from probe sets with absent calls, and removing arrays that were not statistically significant (see Methods section).

Upon ER stress, a large portion of transcripts showed reduced association with polysomes representing global translation repression with increased eIF2~P (Fig. 7A). mRNA transcripts corresponding to ~750 probe sets (<0.05 p-value) showed 10% or greater association with large polysomes during ER stress compared to no stress. Total mRNA levels for many genes were not significantly changed in response to ER stress, while changes in their translation efficiencies were observed. This suggests that translation control can be important for determining gene expression levels. The functions of the top 200 genes suggested to be subject to preferential translation upon ER stress are varied, with almost twenty percent involved in transcription (Fig. 7C). Additionally, these genes are involved in other modes of general expression, including mRNA processing, and protein synthesis and degradation. Finally, DNA repair and metabolism processes are also suggested to be affected by the preferential translation.

Together, this suggests that preferential translation has a significant role in the reprogramming of gene expression, as well as other key cellular processes that can affect the health of the cell.

Included among the transcripts that are preferentially associated with large polysomes in response to ER stress are *ATF4* and *ATF5*, which have been reported to be preferentially translated. In our microarray analysis we also identified *CHOP* having greater association with large polysomes in response to ER stress. As discussed earlier, *CHOP* is critical for the eIF2~P induced ISR. While the transcriptional regulation of *CHOP* during ER stress is well characterized, the contribution of translational expression of *CHOP* is not well defined. In this thesis research, I addressed the mechanisms regulating *CHOP* mRNA translation in response to eIF2~P, and the role this preferential translation can play in cell survival in response to cellular stresses.

2. eIF2~P is required for *CHOP* transcription and translation

In response to ER stress, eIF2~P triggers preferential translation of *ATF4* mRNA concurrent with repressed global translation initiation. This is illustrated by treatment of MEF cells with thapsigargin, a potent ER stress agent (1). Within 1 hour of thapsigargin exposure, wild-type MEF cells displayed an enhanced eIF2~P accompanied by increased expression of ATF4 protein (Fig. 8A). By contrast, MEF cells containing Ala for the eIF2α phosphorylation site Ser-51 (A/A) showed no eIF2~P and minimal levels of ATF4 protein. In addition to translational control, *ATF4* was reported to be subject to transcriptional regulation, with a 3-fold increase in *ATF4* mRNA following 6 hours of the

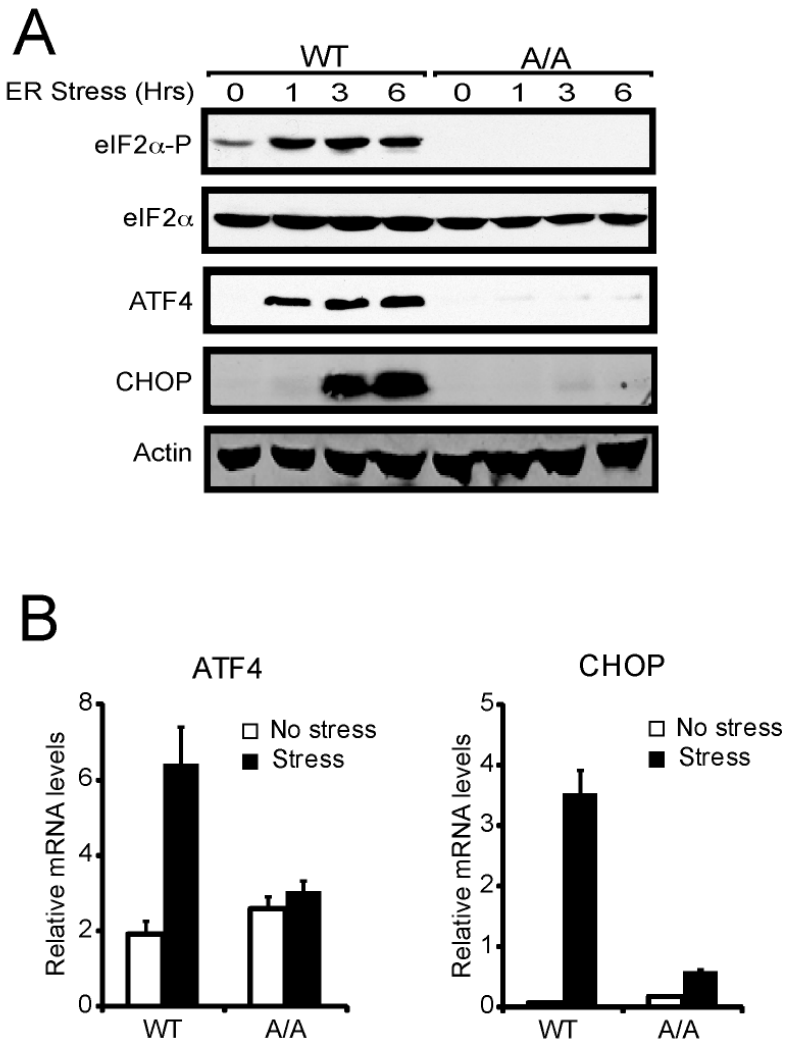
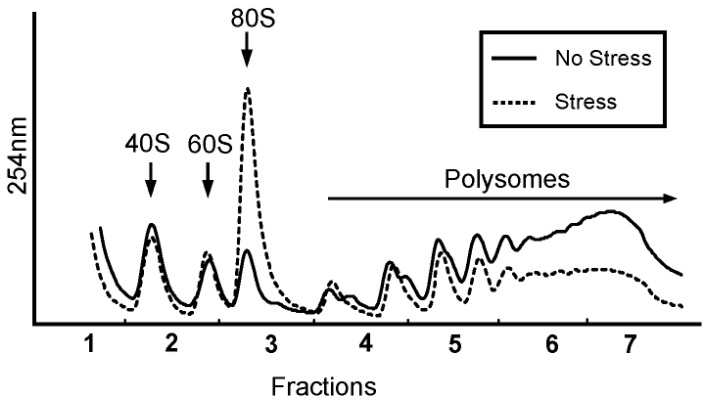
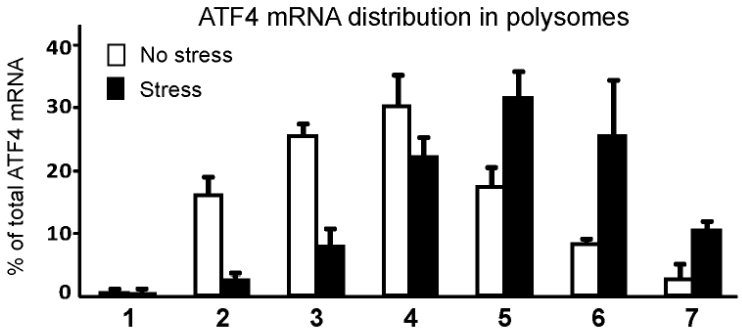
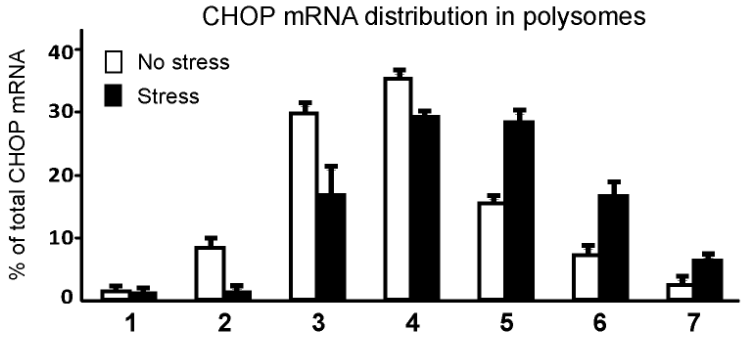


Figure 8. Phosphorylation of eIF2 Increases *CHOP* expression in response to ER stress. (A) Wild-type MEF cells (WT) and mutant cells expressing the non-phosphorylated eIF2α-S51A (A/A) were treated with the ER stress agent, thapsigargin, for up to 6 hours, as indicated, or to no stress treatment (0 hours). Lysates were prepared and the levels of phosphorylated eIF2α, total eIF2α, ATF4, CHOP, and β-actin were measured by immunoblot analysis using antibody that specifically recognizes each protein. (B) Total RNA was isolated from the wild-type and A/A MEF cells treated with

thapsigargin for 6 hours (stress), or to no stress, and the relative levels of *ATF4* and *CHOP* mRNA were measured by qRT-PCR.


80S
No Stress
Stress
40S
60S
254nm
Polysomes
1
2
3
4
5
6
7
Fractions


ATF4 mRNA distribution in polysomes
No stress
Stress
% of total ATF4 mRNA
40
30
20
10
0
1
2
3
4
5
6
7


CHOP mRNA distribution in polysomes
No stress
Stress
% of total CHOP mRNA
40
30
20
10
0
1
2
3
4
5
6
7

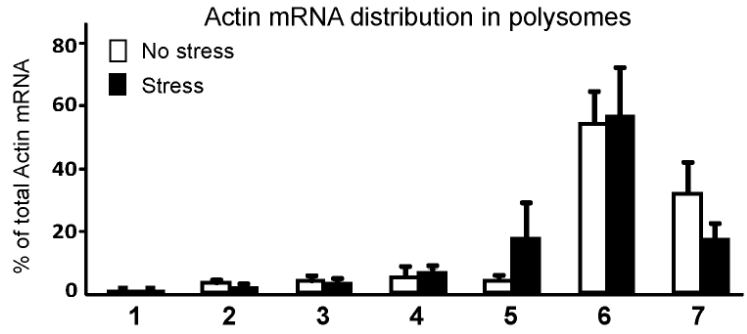

Actin mRNA distribution in polysomes
No stress
Stress
% of total Actin mRNA
80
60
40
20
0
1
2
3
4
5
6
7

Figure 9. Both *ATF4* and *CHOP* mRNAs are preferentially associated with large polysomes during ER stress. Wild-type MEF cells were exposed to the ER stress agent thapsigargin for 6 hours (stress), or no stress treatment. Cell lysates were then analyzed by centrifugation in a 10% to 50% sucrose gradient, and the profiles were measured by absorbance at 254 nm. The top figure highlights the 40S and 60S ribosomal subunits, 80S monosomes, and polysomes. Total RNA was prepared from the fractions collected from the sucrose gradients, and the percentage of *ATF4*, *CHOP*, and actin-encoding transcripts present in each of the seven fractions derived from the ER stress (Stress) or the non-treated cells (No stress) were determined by qRT-PCR. Values are represented as histograms for each fraction. Three independent experiments were carried out for each measurement, with the S.D. indicated for each. The top panel is representative of three independent experiments.

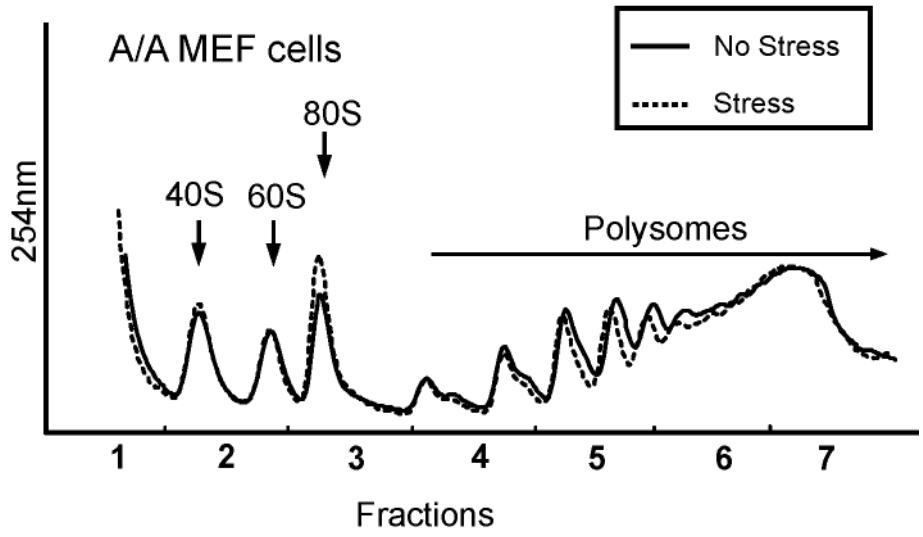


Figure 10. Repression of translation initiation does not occur in A/A MEF cells in response to ER stress. A/A MEF cells expressing the mutant eIF2α-S51A were treated with the ER stress agent thapsigargin for 6 hours (Stress), or no stress treatment. Cell lysates were then analyzed by centrifugation in a 10% to 50% sucrose gradient, and the elution fractions were measured by absorbance at 254 nm. The 40S and 60S ribosomal subunits, 80S monosomes, and polysomes are indicated.

ER stress (Fig. 8B) (162). This increase in *ATF4* mRNA levels in response to ER stress is substantially blocked in the A/A cells.

ATF4 is a transcriptional activator of ISR genes, such as *CHOP*. Levels of *CHOP* protein and mRNA were sharply increased in response to ER stress by a mechanism requiring eIF2~P (Fig. 8A and 8B). Given that increased expression of CHOP protein occurs despite high levels of eIF2~P, we reasoned that translation of *CHOP* mRNA may be favored even when global translation initiation is severely restricted. This idea was supported for our genome-wide analysis of mRNAs that showed enhanced association with large polyribosomes in response to ER stress (Fig. 7). To further test this idea, we carried out a polysome analysis using sucrose gradient centrifugation. As noted above, thapsigargin treatment of MEF cells significantly reduces polysome levels, concomitant with elevated levels of free ribosomes and monosomes, which is consistent with repressed translation initiation (Fig. 9). This reduction in translation initiation is dependent on eIF2~P, as the polysome profile was largely unchanged when A/A cells were treated with thapsigargin (Fig. 10).

During non-stressed conditions the levels of *ATF4* transcript, measured as the percentage of total *ATF4* mRNA, were most abundant in the monosome and small polysome fractions of the sucrose gradient. In this condition, only 28% of *ATF4* mRNAs were associated with large polysomes consisting of transcripts associated with four or more ribosomes. By comparison, upon ER stress, there was a substantial shift of *ATF4* transcripts to the large polysome fractions (67% associated with large polysomes), consistent with earlier reports that *ATF4* mRNA is preferentially translated upon eIF2~P. *CHOP* mRNA displayed a similar distribution pattern in the polysome profiles as the

ATF4 transcripts (Fig. 9). In the absence of stress, *CHOP* mRNA was most abundant in the monosomes and small polysomes, while ER stress triggered increased association of this transcript with the large polysomes (25% associated with large polysomes in non-stressed conditions compared to 52% during ER stress). As a control, we also measured β-actin mRNA among the fractions in the sucrose gradient and found that this transcript was largely insensitive to ER stress.

3. *CHOP* translational control is facilitated by an uORF in the 5'-leader of the *CHOP* mRNA

We next addressed whether the 5'-leader of the *CHOP* mRNA directs translational control in response to ER stress. The transcriptional start site of the *CHOP* transcript was determined in MEF cells in the presence or absence of stress by 5'-RACE and DNA sequencing (Fig 11A). Transcription of *CHOP* occurs at the same site independent of stress conditions, leading to a 5'-leader sequence 162 nucleotides in length. The *CHOP* leader sequence encodes a single uORF representing a 34-residue polypeptide that is highly conserved among vertebrates (Fig. 11B) (163). Notable among the conserved residues are Met residues at positions 1 and 4 (encoded by codons designated ATG1 and ATG2), providing for two possible initiation codons, and basic amino acid residues in the carboxy terminus of the uORF.

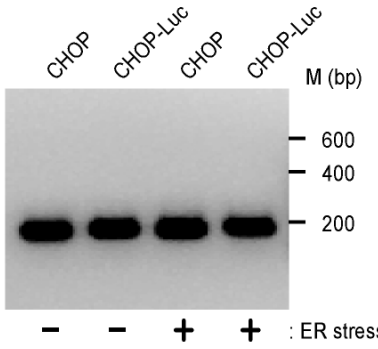
The role of the 5'-leader sequence of the *CHOP* transcript in translation control was investigated by using a P_{TK}-*CHOP-Luc* reporter, which contained a cDNA segment encoding the mouse *CHOP* 5'-leader segment fused to firefly luciferase downstream of the minimal *TK* promoter. *CHOP-Luc* expression was increased by 3-fold in the wild-

type MEF cells in response to ER stress (Fig. 11C). In contrast, there was low luciferase activity in the A/A cells devoid of eIF2~P, which did not appreciably change in response to ER stress. The transcriptional start site of the a P_{TK}-*CHOP-Luc* reporter was the same as that determined for the endogenous *CHOP* (Fig. 11A), and there were no significant changes in *CHOP-Luc* mRNA levels in the tested conditions, consistent with the 5'-leader of the *CHOP* mRNA directing translational expression in response to eIF2~P (Fig. 11C).

Earlier studies suggested that translation of the uORF can repress translation of the downstream *CHOP* coding region (163). In order to identify the underlying mechanisms by which eIF2~P and stress leads to preferential translation of *CHOP*, we constructed a series of mutations in the 5'-leader portion of the P_{TK}-*CHOP-Luc* reporter and analyzed their effects on expression in response to ER stress. First, we addressed whether *CHOP* mRNA translation initiation occurs by cap-dependent ribosome scanning or is rather facilitated by an internal translation initiation process such as IRES-mediated initiation (6, 164). A stem-loop structure was inserted 5'- to the uORF (Fig. 12). Minimal *CHOP-Luc* expression was observed in MEF cells irrespective of stress conditions (Fig. 12). This result supports the idea that *CHOP* translation involves the processive scanning of ribosomes from the 5'-end of the *CHOP* transcript.

We next mutated the ATG1 and ATG2 of the uORF to AGG, individually or in combination, in the P_{TK}-*CHOP-Luc* reporter. Mutation of ATG1 led to elevated *CHOP-Luc* expression during both stress and non-stressed conditions compared to the wild-type reporter (Fig. 12). The increase was greatest in the non-stressed conditions, with over a 3-fold increase in *CHOP-Luc* expression in the ΔATG1 mutant compared to the wild-type

A




```
  1  tttctgattg gtaggctcct ggaccctgcc ccccccccag gaggggccgg
 51  acagcataaa ggatactcgc tctccgctct tgaggtcaGT TATCTTGAGC
                                          uORF
101  CTAACACGTC GATTATATCA TGTTGAAGAT GAGCGGGTGG CAGCGACAGA
          120 nt                  G          G
151  GCCAGAATAA CAGCCGGAAC CTGAGGAGAG AGTGTTCCAG AAGGAAGTGC

201  ATCTTCATAC ACCACCACAC CTGAAAGCAG AACCTGGTCC ACGTGCAGTC
                                  G
     CHOP          Luciferase
251  ATGGCAGCTA TGGAAGACGC CAAAAACATA AAGAAAGGCC
```

B

CHOP uORF sequences

```
Human      MLKMSG------------WQRQSQNQSWNLRRECSRRKCIFIHHHT 34
Mouse      MLKMSG------------WQRQSQNNSRNLRRECSRRKCIFIHHHT 34
Rat        MLKMSG------------WQRQSQNNSRNLRRECSRRKCIFIHHHT 34
Hamster    MLKMNG------------WQRQSQNKSRDLRSECSRRKCIFIHHHT 34
Pig        MLKMSR------------WQRQSQNQ-RNLRRECSRRKCIF-HHHT 32
Bear       MLKMSG------------WQPQSQNQSRNLRRECSRRKCIF-HHHT 33
Cow        MLKMSG------------WQRQSQNQSRNLRRECSRRKCIFIHHHT 34
Sheep      MLKMSG------------WQRQSQNQ-RNLRRECSRRKCIFIHHHT 33
Dog        MLKMSG------------WQPQSHSQSRNLRRECSRRKCIFIHHHT 34
Frog       MFNMSPQSLNVQPQTSSRCRFICQKRTDPTWRKCWRRKTIFIHHHT 46
Fish       MVNMSDQPSL-QKHTQTLNQKQPRKRNNKKKRSYWDKISPTTHIQT 45
Consensus  MlkMs             wq qsq     lrrecsrrkcif HhhT
```

C

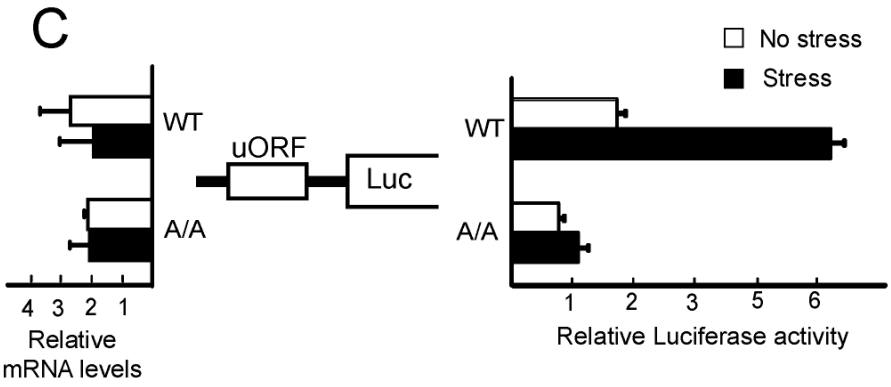


Figure 11. The 5'-leader of the *CHOP* mRNA contains an uORF that is required for translational control in response to eIF2~P. (A, Top panel) 5'-RACE was carried out for endogenous *CHOP* and *CHOP-Luc* using RNA prepared from wild-type MEF cells expressing the P_{TK}-*CHOP-Luc* reporter, which were treated with the ER stress agent thapsigargin (+), or no stress agent (16). DNA products were separated and visualized by electrophoresis using a 1.2% agarose gel, with markers of the indicated size in base-pairs represented on the right. (A, bottom panel) The sequence of the 5'-leader of the mouse *CHOP* mRNA is presented with the boxes indicating the uORF and the coding region of the CHOP-luciferase fusion. Residues mutated in the analysis of *CHOP* translational control are indicated below the box. The bold arrow indicates the transcription start site of the *CHOP* gene as determined by sequencing of the 5'-RACE products. A stem-loop structure or 120-bp segment was inserted at the indicated position upstream of the uORF. (B) The polypeptide sequences encoded by the uORF in the *CHOP* mRNAs from different vertebrates. The uORF polypeptide sequences were from representative cDNAs derived from the indicated *CHOP* orthologs, including human (GenBank accession number BC003637), mouse (BC013718), rat (BC100664), hamster (M29238), pig (AK346731), bear (GW278660), cow (BC122721), sheep (DY499855), dog (DN431044), frog *Xenopus tropicalis* (BC153679), and fish *Danio rerio* (BC134052). The number of polypeptide residues encoded by each uORF is listed following the sequences. Residues conserved among the uORF sequences are listed in the consensus, with invariant residues in capital letters and those conserved in small case letters. (C) *CHOP* translational control in response to ER stress was measured by a dual luciferase assay. The P_{TK}-*CHOP-Luc* reporter and a Renilla luciferase plasmid, which served as an

internal control, were transfected into wild-type MEF cells (WT) or A/A cells expressing eIF2α-S51A, and treated with thapsigargin (Stress), or no stress. The P_{TK}-*CHOP-Luc* reporter contains cDNA sequences corresponding to the entire 5'-leader of the *CHOP* mRNA, which is illustrated along with the luciferase reporter gene. Three independent experiments were carried out for each measurement, and relative values are represented as histograms for each, with the S.D. indicated. In parallel, the levels of the *CHOP-Luc* mRNA were measured by qRT-PCR, and relative values of the reporter transcripts were presented as histograms, with error bars representing the S.D..

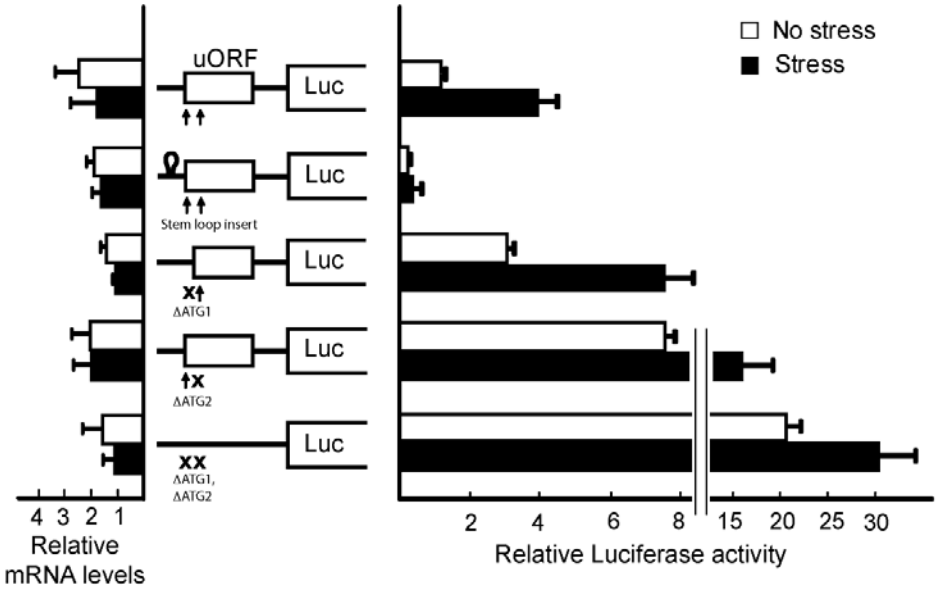
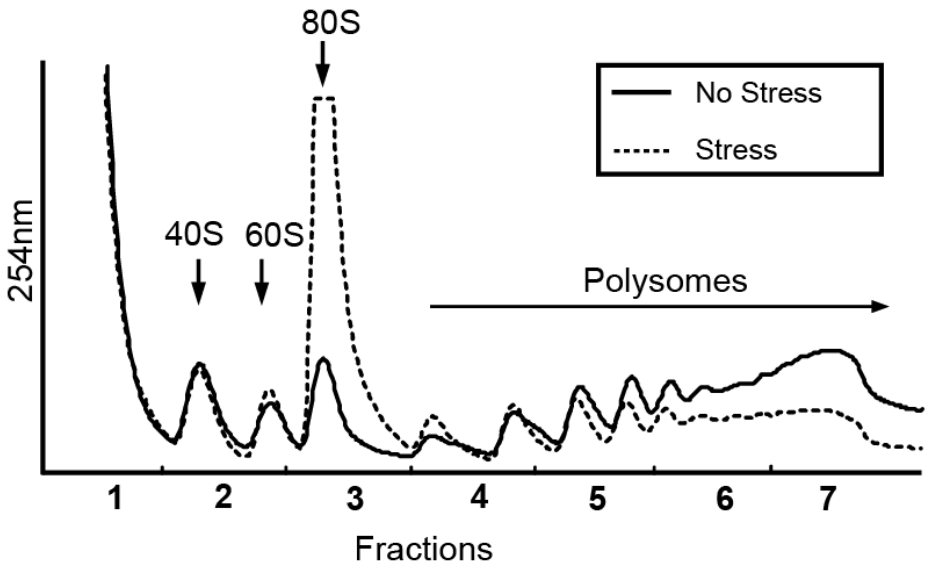


Figure 12. The uORF is inhibitory to *CHOP* translation. The illustrated wild-type and mutant versions of the P_{TK}-*CHOP-Luc* reporter were transfected into wild-type MEF cells, and dual luciferase assays were carried out in response to thapsigargin (stress), or no stress. Mutant versions of the *CHOP-Luc* reporter include an insertion of a stem-loop structure upstream of the uORF, and "X" indicates ATG1 and ATG2 of the uORF substituted to AGG, individually or combined. Additionally, the relative amounts of *CHOP-Luc* mRNA were measured by qRT-PCR. Three independent experiments were carried out for each of the measurements, and relative values are represented as histograms, in conjunction with the indicated S.D..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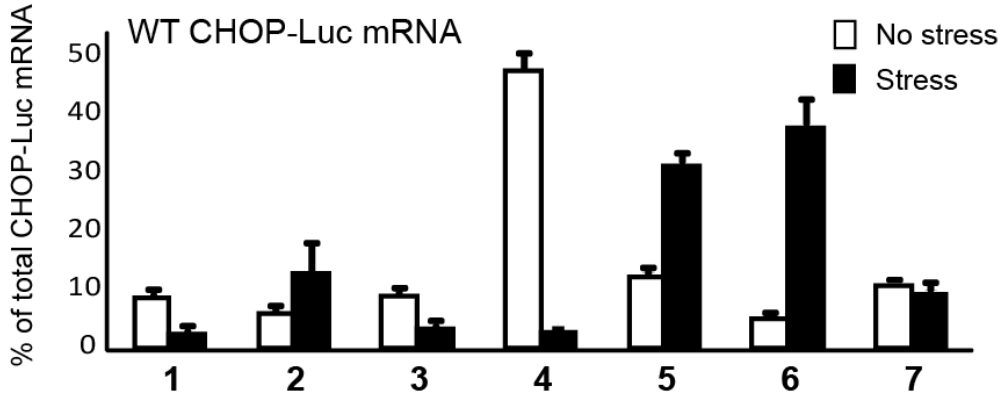
No Stress
Stress
40S
60S
254nm
Polysomes
1
2
3
4
5
6
7
Fractions


WT CHOP-Luc mRNA
No stress
Stress
% of total CHOP-Luc mRNA
50
40
30
20
10
0
1
2
3
4
5
6
7

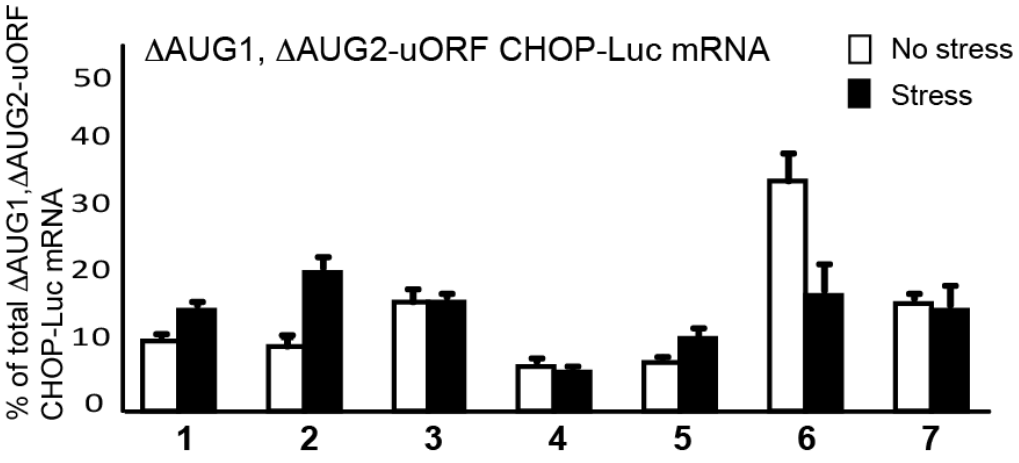

ΔAUG1, ΔAUG2-uORF CHOP-Luc mRNA
No stress
Stress
% of total ΔAUG1,ΔAUG2-uORF CHOP-Luc mRNA
50
40
30
20
10
0
1
2
3
4
5
6
7

Figure 13. *CHOP-Luc* mRNA is preferentially associated with large polysomes in response to ER stress. Wild-type MEF cells were transfected with a wild-type version of the P_{TK}-*CHOP-Luc* reporter, or a version with mutations in both ATG1 and ATG2 (ΔATG1 and ΔATG2). Transfected cells were exposed to thapsigargin (Stress) for 6 hours, or to no stress treatment. Cell lysates were then analyzed by sucrose gradient centrifugation and fractions were monitored by absorbance at 254 nm (Top figure), with the indicated 40S and 60S ribosomal subunits, 80S monosomes, and polysomes. Total RNA was prepared from the fractions, and the percentage of *CHOP-Luc* mRNA present in each of the seven fractions obtained from the ER stress (Stress) or the non-treated cells (No stress) was determined by qRT-PCR. Three independent experiments were carried out, with measurements for each fraction illustrated, along with the S.D. values.

reporter, while during ER stress there was about a 2-fold elevation (Fig. 12). Together, these results indicate a diminished induction during ER stress, with about a 2-fold enhancement in the MEF cells expressing the ΔATG1 mutant and a 4-fold increase in the wild-type version. Loss of ATG2 led to an even higher increase in *CHOP-Luc* expression compared to the wild-type counterpart, with increases of 7- and 4-fold during the non-stressed and stressed conditions, respectively. Furthermore, the highest luciferase activity was observed when both ATG1 and ATG2 were mutated (Fig. 12). Levels of *CHOP-Luc* mRNA were comparable between the different ATG mutant arrangements and stress arrangements (Fig. 12). These results support the idea that both ATG1 and ATG2 are able to serve as initiation codons for the uORF, with ATG2 being predominant.

The uORF is suggested to be inhibitory to *CHOP* translation, and this repressing function can be overcome with stress and eIF2~P. Further supporting this model, the wild-type *CHOP-Luc* mRNA was found to be preferentially associated with large polysomes in MEF cells treated with thapsigargin, while this transcript was the most abundant in the disome fraction in the absence of stress (Fig. 13). By comparison, mRNA expressed from the P_{TK}-*CHOP-Luc* reporter devoid of both ATG1 and ATG2 was most prevalent in polysome fraction 6 of the sucrose gradient in the absence of stress. During ER stress, the *CHOP-Luc* transcript was less abundant in the large polysome fractions, with a broad distribution among several fractions of the sucrose gradient (Fig. 13).

4. *CHOP* translational control is mediated by leaky scanning of ribosomes through the inhibitory uORF

We considered two models by which stress and eIF2~P can overcome the inhibitory functions of the uORF in *CHOP* translational control. The first is a "Reinitiation" model, which suggests that following translation of the inhibitory uORF during low eIF2~P, ribosomes dissociate from the *CHOP* mRNA, and therefore *CHOP* expression is repressed. Upon stress and eIF2~P, ribosomes translating the uORF would resume scanning and re-initiate translation at the *CHOP* coding region. Alternatively, in a "Bypass" model, ribosomes initiate translation at the uORF in non-stressed conditions. In this latter model, translation of the uORF would preclude expression at the downstream *CHOP* coding region. In response to stress and increased eIF2~P, the scanning ribosome would bypass or scan through the inhibitory uORF and instead initiate translation at the downstream *CHOP* coding region. To delineate between these two models, we mutated the stop codon of the *CHOP* uORF to a sense codon (TGA to GGA), resulting in an extended uORF that overlaps by 19 nucleotides out-of-frame with the CHOP coding region (Fig. 11A and 14). Luciferase activity from the P_{TK}-*CHOP-Luc* with the extended uORF was induced in response to ER stress similar to that measured for the wild-type reporter. This result strongly supports the Bypass model, as the extended uORF would not be expected to interfere with the induced *CHOP* translation. By contrast, in the Reinitiation model ribosomes terminating translation of the extended uORF would be 3'-of the initiation codon of the *CHOP* coding region and require protracted 3' to 5' scanning to express CHOP, an unlikely event.

We next addressed the basis for the ribosome bypass of the uORF in response to eIF2~P. We considered two ideas for the ribosome bypass: the poor initiation context of ATG1 and ATG2 encoded in the uORF, and the short length (31 nucleotides) from the 5'-end of the *CHOP* mRNA to the AUG1 of the *CHOP* uORF, both of which may reduce translation of the uORF during eIF2~P. Start codon context is an important contributor to the efficiency of translation initiation (165-167). The uORF of *CHOP* has a less than optimal start codon context at ATG1 (TATATC**ATG**T) and the primary ATG2 (TTGAAG**ATG**A) compared to the Kozak consensus sequence, gcc(A/G)cc**ATG**G, where the capital letters at -3 and +4 in the consensus are most critical for translation initiation. The translation initiation context of *ATF4* uORF1 matches this consensus (GCCACC**ATG**G), and this sequence was substituted into the *CHOP* uORF, replacing the predominant ATG2 in the absence of ATG1 in the P_{TK}-*CHOP-Luc* reporter (Fig. 14). Substitution of the strong Kozak consensus led to significantly lowered *CHOP-Luc* expression in response to ER stress, with about a 2-fold reduction compared to the wild-type reporter. Levels of *CHOP-Luc* mRNA were comparable between the Kozak consensus and its wild-type counterpart (Fig. 12). These results suggests that less optimal initiation site contexts in the uORF contribute to leaky scanning, a mechanism by which the inhibitory uORF is bypassed during stress conditions and high levels of eIF2~P.

To determine whether the abbreviated leader length preceding the uORF is important for stress-induced *CHOP* translation, a heterologous 120-nucleotide sequence (79), devoid of any start and stop codons and without predicted strong secondary structures, was inserted upstream of the uORF in the P_{TK}-*CHOP-Luc* reporter (Fig. 14). Insertion of this sequence did not change the regulation of *CHOP-Luc* expression, with a

4-fold increase in luciferase activity in response to ER stress, which was similar to the wild-type reporter (Fig. 14). There were also no significant changes in *CHOP-Luc* mRNA levels with the 120-nucleotide insertion. These results indicate that bypass of the inhibitory uORF in response to stress is not dependent upon the relatively short length of *CHOP* mRNA situated between the 5'-end and the initiation codons of the uORF.

5. eIF1 facilitates ribosome bypass of inhibitory uORF and enhances CHOP translation

eIF1 plays key role in translation, promoting initiation at AUG codons that are closest to the Kozak consensus (17, 157). As previously described, the uORF of *CHOP* has a less than optimal start codon context at ATG1 and ATG2 compared to the Kozak consensus sequence. To test the Bypass model we investigated whether cells over-expressing eIF1 prefer initiation codons at the uORF or the *CHOP* coding region for translation initiation. eIF1 was co-expressed with *p-TK-CHOP-Luc* in MEFs in the presence or absence of stress. As expected enhanced *CHOP-Luc* expression was observed with over-expression of eIF1 independent of stress compared to pcDNA vector control (Fig. 15A). There was no change in the stress-induction ratio in CHOP-Luc expression when eIF1 was co-expressed with the reporter.

Next we measured endogenous CHOP levels in cells that over-expressed eIF1. Cells transfected with the eIF1 expression plasmid, or pcDNA vector alone, were treated with 0.5 μM thapsigargin for up to 2 hours. There was an increased basal, as well as stress-induced CHOP protein levels when eIF1 was over-expressed compared to the pcDNA control (Fig. 15). Interestingly, the levels of *CHOP* mRNA during ER stress

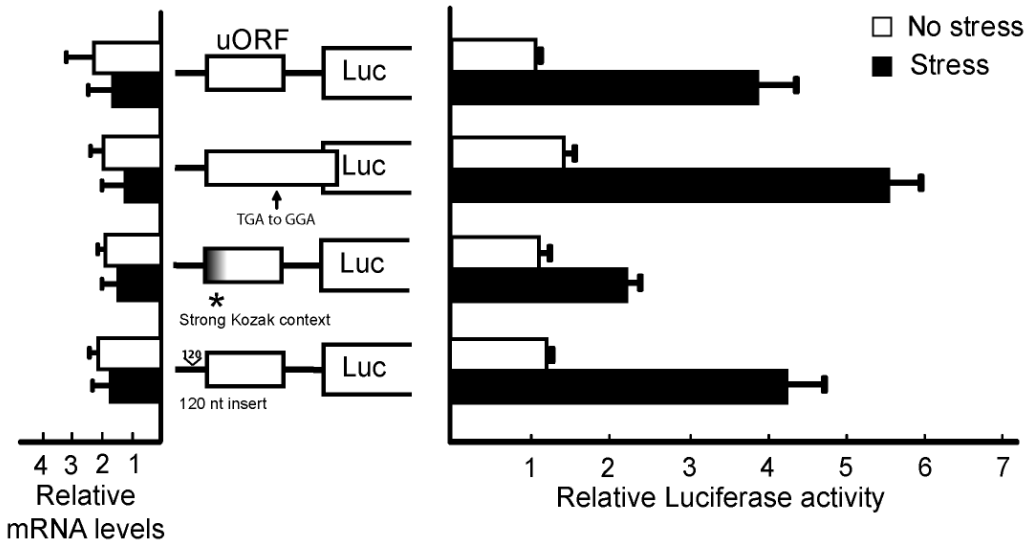


Figure 14. A strong start codon context for initiation of uORF translation thwarts bypass of the inhibitory element in response to ER stress. Wild-type and the indicated mutant versions of the P_{TK}-*CHOP-Luc* reporter were transfected into wild-type MEF cells, and dual luciferase assays were carried out in response to thapsigargin (Stress), or no stress. Mutant versions include substitution of the encoded stop codon of the uORF (TGA to GGA), leading to an extension of the uORF so that it over-laps out-of-frame with the *Luc* coding region. Additionally, a strong Kozak context was substituted for ATG2 of the uORF, in the absence of ATG1, and a 120-nucleotide segment devoid of initiation codons and strong predicted secondary structure were inserted upstream of the uORF. The relative amounts of *CHOP-Luc* mRNA were also measured by qRT-PCR. Three independent experiments were carried out for each assay, and the relative values are represented with the S.D..

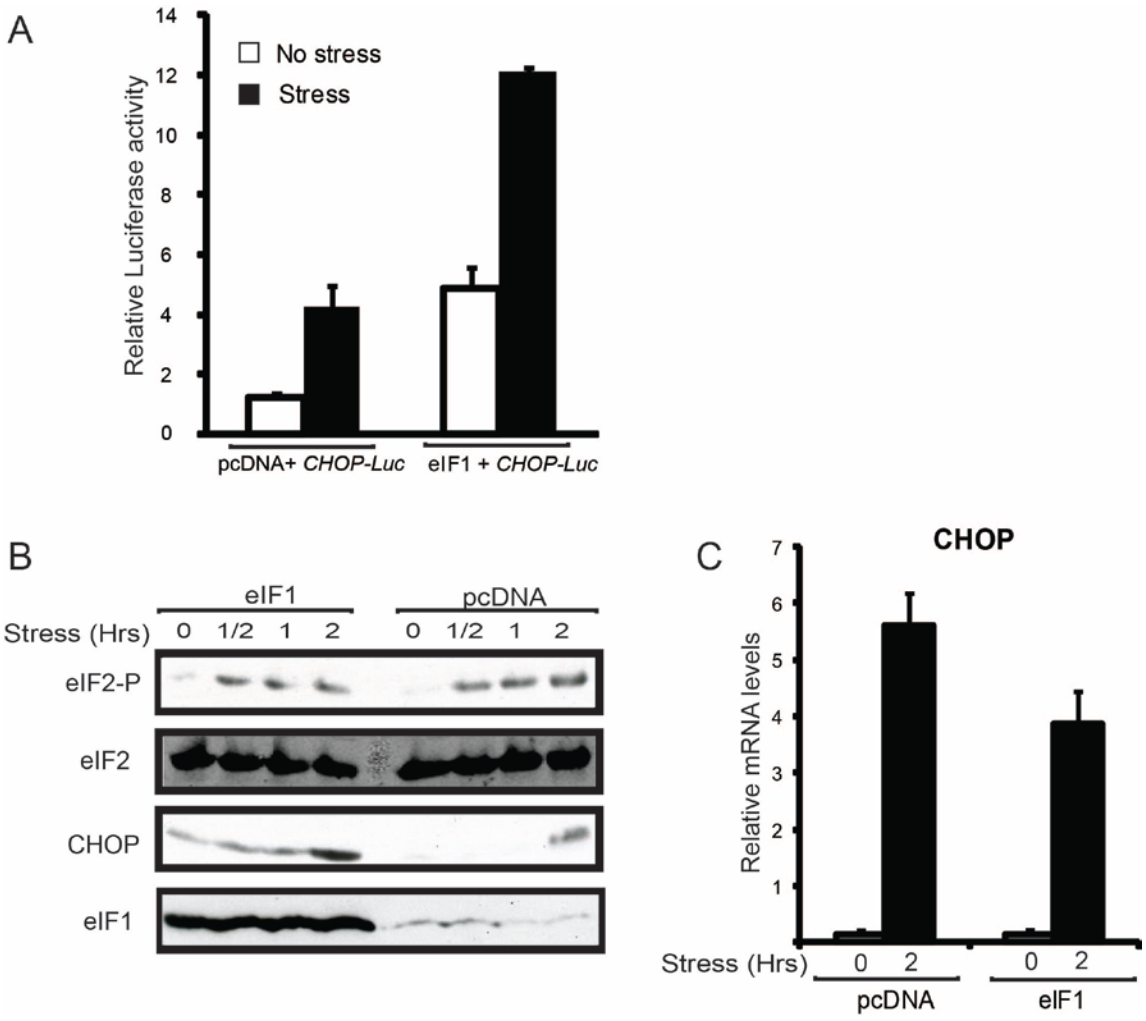


Figure 15. Over-expression of eIF1 facilitates ribosome bypass of the inhibitory upstream ORF and enhances *CHOP* expression. (A) The P_{TK}-*CHOP-Luc* plasmid, encoding an in-frame fusion between the CHOP 5'leader and firefly luciferase, was co-transfected with plasmid DNA encoding eIF1 or pcDNA into wild-type MEF cells, and dual reporter assays were carried out in response to the ER stress agent thapsigargin, or during no stress. (B) MEF cells were transfected with pcDNA or plasmid DNA encoding eIF1 were treated with ER stress for up to 2 hours, as indicated, or to no stress treatment (0 hours). Lysates were prepared and the levels of phosphorylated eIF2α, total eIF2α, CHOP, and eIF1 were measured by immunoblot analysis using antibody that specifically

recognizes each protein. (C) RNA was isolated from the wild-type MEF cells over-expressing eIF1, or containing vector alone, and the relative levels *CHOP* mRNA were measured by qRT-PCR.

were lower with over-expression of eIF1 compared to the pcDNA control (Fig 15). These results support the idea that eIF1 facilitates the ribosome bypass of the inhibitory uORF that has initiation codons in poor Kozak contexts, leading to enhanced *CHOP* expression.

6. The carboxy-terminal portion of the uORF is inhibitory to the downstream *CHOP* ORF translation

We next investigated the mechanism by which the uORF represses translation of the downstream *CHOP* coding region. The basis for the inhibitory function of the uORF in the Bypass model may be that following termination of the uORF translation ribosomes would dissociate from the *CHOP* mRNA; alternatively translation of the uORF may lead to a block in translation elongation or termination that prevents translation at the downstream *CHOP* ORF. Arrested ribosomes may not only stall synthesis of the nascent polypeptide, but also impede subsequent scanning from the 5'-end of the *CHOP* mRNA. To address the inhibitory properties of the uORF in translation we constructed an in-frame fusion between the uORF and firefly luciferase expressed from the TK promoter. We transfected the resulting P_{TK}-*uORF-Luc* plasmid into wild-type MEF cells, and found minimal luciferase activity and no detectable fusion protein as judged by immunoblot analyses (WT in Fig. 16A and B). Deletion analysis of the uORF portion of the fusion gene, including an in-frame deletion of uORF codons 14 to 34 (Δ14-34) and 24 to 34 (Δ24-34), led to significant increases in the luciferase activity and measureable fusion proteins by immunoblot. By comparison, removal of the uORF codons 14 to 23 (Δ14-23) showed low uORF-Luc expression, as judged by luciferase activity and an immunoblot measurement of the fusion protein (Fig. 16A and B). The

levels of *uORF-Luc* mRNA were similar among the MEF cells expressing the fusion protein with the full-length uORF and the deletion mutants (Fig. 16A). These results support the idea that the 3'-portion of the uORF, including codons 24 to 34, can block translation.

The uORF was next truncated in the P_{TK}-*CHOP-Luc* reporter by introducing a stop codon at residue 24 (AGA to TGA) (Fig. 11A and 16C). Expression of the *CHOP-Luc* was significantly increased during both the stressed and non-stressed conditions compared to the wild-type reporter (Fig. 16C). These results suggest that translation of the carboxy-terminal portion of the uORF can lead to a block in translation elongation or termination, which can effectively prevent subsequent initiation at the downstream *CHOP* coding region. Removal of the inhibitory portion of the uORF is suggested to allow for a significant amount of the ribosomes translating the uORF to resume scanning along the mRNA and reinitiate at the downstream *CHOP* coding region.

We previously observed that optimizing the Kozak context for ATG1 and ATG2 in the uORF prevents bypass of the inhibitory uORF in response to ER stress (Fig. 14). Taking this into consideration, we further reasoned that if the inhibitory portion of the uORF was removed that this would at least in part overcome the inclusion of the optimized initiation codon context in the P_{TK}-*CHOP-Luc* reporter. This was indeed the outcome with elevated levels of *CHOP-Luc* expression in the MEF cells during non-stressed conditions that were similar to that measured in the ER stressed MEF cells expressing the wild-type reporter (Fig. 16C). Note that during ER stress luciferase activity expressed from the P_{TK}-*CHOP-Luc* reporter containing the combined Kozak consensus and Δ24-34 deletion did not match that with the carboxy terminal deletion

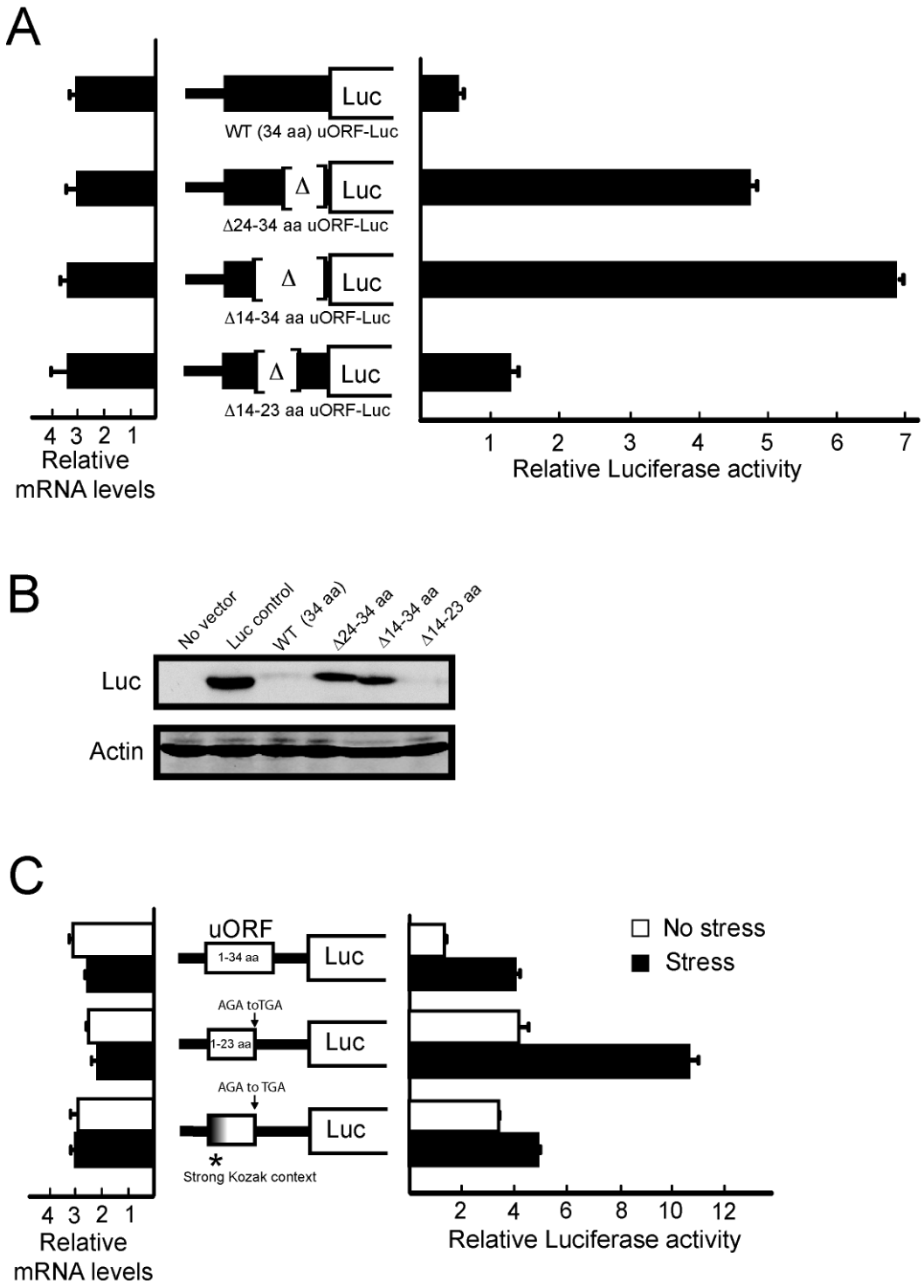


Figure 16. The carboxy terminal portion of the uORF is inhibitory to *CHOP* translation. (A) The P_{TK}-*uORF-Luc* plasmid, encoding an in-frame fusion between the

uORF and firefly luciferase, was transfected into wild-type MEF cells, and dual reporter assays were carried out for each. The P_{TK}-*uORF-Luc* plasmid contained the full-length uORF, or versions containing deletions of codons 24 to 34, 14 to 34, or from 14 to 23, as illustrated. In parallel, the relative amounts of *CHOP-Luc* mRNA were also measured by qRT-PCR. Three independent experiments were carried out for each of the reporter construct, and the relative values are represented for each, with the S.D. indicated. (B) The levels of the uORF-Luc fusion protein containing the full-length uORF, or the indicated deletion mutants, were determined by immunoblot analysis using antibody that recognizes firefly luciferase. Actin levels were also measured for normalization between the lysates. As control, the immunoblot analysis was also carried out with lysates prepared from MEF cells containing no vector or with vector expressing only firefly luciferase. (C) The illustrated wild-type and mutant versions of the P_{TK}-*CHOP-Luc* reporter were transfected into wild-type MEF cells, and dual luciferase assays were carried out in response to ER stress, or during no stress. The mutant versions include a mutation of codon 24 (AGA) of the uORF to TGA to generate the luciferase reporter with shortened version of uORF. Additionally, the shortened version of uORF was combined with the strong Kozak context, as illustrated. Relative levels of *CHOP-Luc* mRNA were also determined by qRT-PCR. Three independent experiments were conducted for each, and the relative values are represented, along with the S.D..

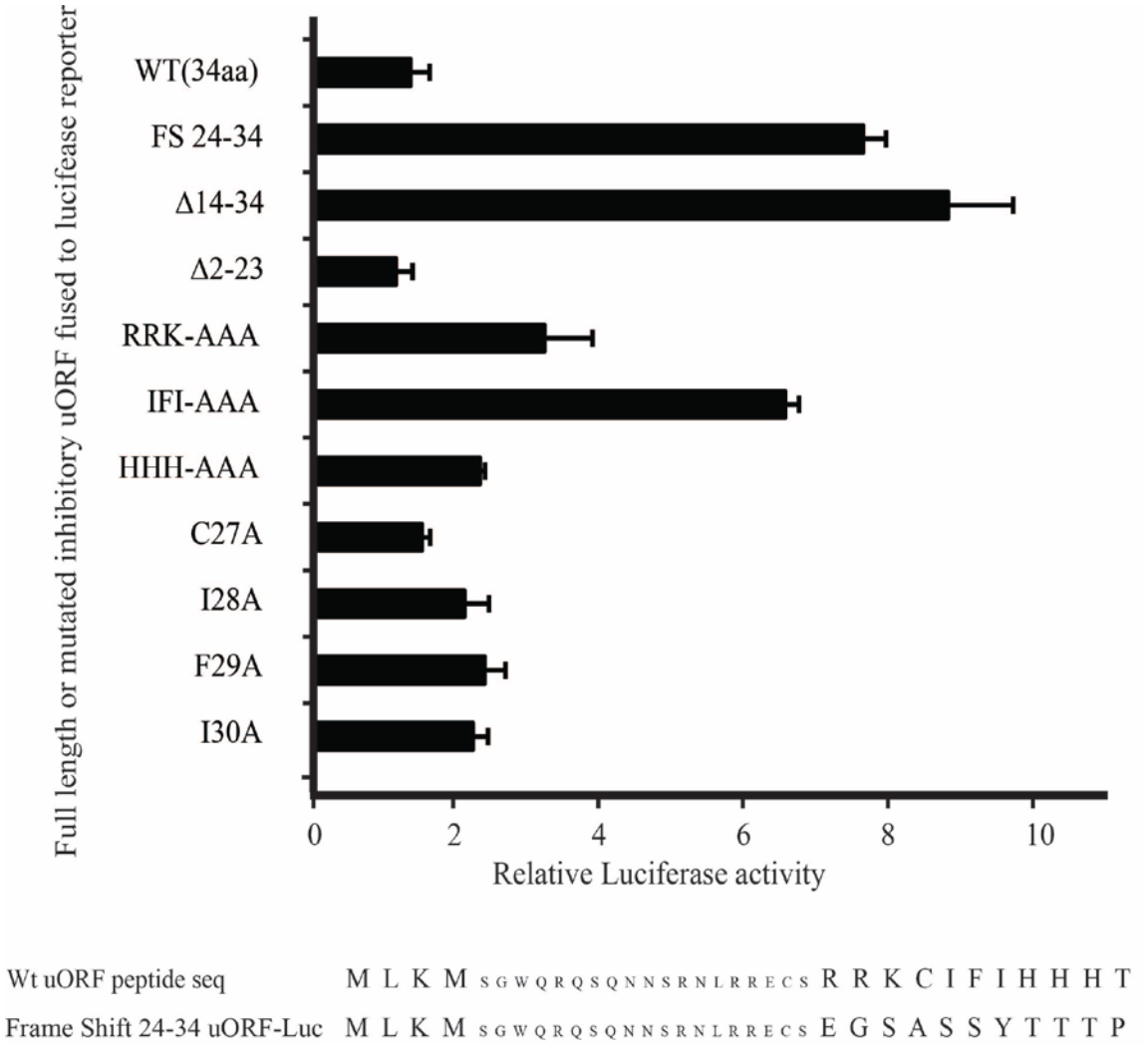


Figure 17. The carboxy-terminal region of the uORF-encoded peptide is inhibitory to *CHOP* mRNA translation. The *P_{TK}-uORF-Luc* plasmid encoding an in-frame fusion between the uORF and firefly luciferase, was transfected into wild-type MEF cells, and dual reporter assays were carried out for each. The *P_{TK}-uORF-Luc* plasmid contained the full-length uORF, or versions containing deletions of codons 14 to 34, 2 to 23, or mutating the indicated residues to alanine. The *P_{TK}-uORF-Luc* plasmid containing the frame shift following codon 23 in the uORF was obtained by inserting one nucleotide after codon 23 and removing one nucleotide just after the codon 34.

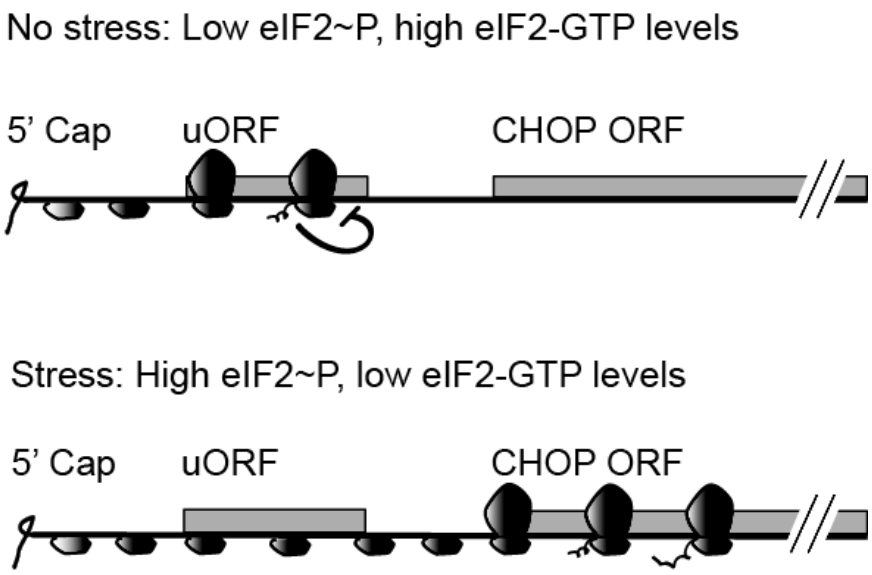


Figure 18. Phosphorylation of eIF2 facilitates ribosome bypass of the inhibitory uORF, enhancing translation of the *CHOP* coding region. In the absence of stress there is low eIF2~P and elevated levels of eIF2-GTP. Ribosomes are suggested to bind the 5'-end of the *CHOP* mRNA, and the scanning ribosomes translate the uORF, leading to a block in translation elongation or termination and low translation of the *CHOP* coding region. During stress, there is robust eIF2~P that reduces exchange of eIF2-GTP to eIF2-GDP that is proposed to reduce translation initiation at the uORF due to the poor initiation codon context. As a consequence, the scanning ribosome bypasses the inhibitory uORF and instead translates the *CHOP* coding region, which has an initiation codon with a strong Kozak consensus.

alone. This suggests that in this stress context, reinitiation at the downstream *CHOP* coding region is limited, and that bypass of the inhibitory uORF in response to eIF2~P is central for preferential translation of *CHOP* mRNA.

7. Enhanced CHOP expression with deletion of the uORF

From the reporter assays and polysome analysis it is evident that the inhibitory uORF is critical for regulating CHOP levels in response to eIF2~P. Here we show that the absence of the uORF indeed increases cellular CHOP levels substantially. Cells derived from *CHOP*$^{-/-}$ MEF cells were transduced to express *CHOP* mRNA with either wild-type uORF or one that has mutations in the initiation codons leading to a deletion of the uORF (ΔuORF). The experimental strategy employed to generate these cell lines involved incorporating a flip recombination target site (FRT) into the genome of *CHOP*$^{-/-}$ cells. First, the FRT site was inserted into the genome of *CHOP*$^{-/-}$ cells using zeomycin/antibiotic selection. A DNA fragment encoding 1-kb of the *CHOP* promoter and the *CHOP* cDNA containing the full-length *CHOP* ORF and 5'-leader sequence including the wild-type uORF or ΔuORF was inserted into FRT containing pcDNA plasmid. The resulting plasmids were co-transfected with flippase encoding plasmid DNA into *CHOP*$^{-/-}$/*FRT* cells. The flippase enzyme facilitates integration of an FRT containing plasmid into the FRT site of *CHOP*$^{-/-}$/*FRT* cells.

The *CHOP*$^{-/-}$ cells that express the *CHOP* cDNA with either the wild-type or deleted uORF were treated with an ER stress agent for up to 6 hours, or no stress. Levels of eIF2~P, ATF4, CHOP and β-actin were measured by immunoblot (Fig. 19). There was

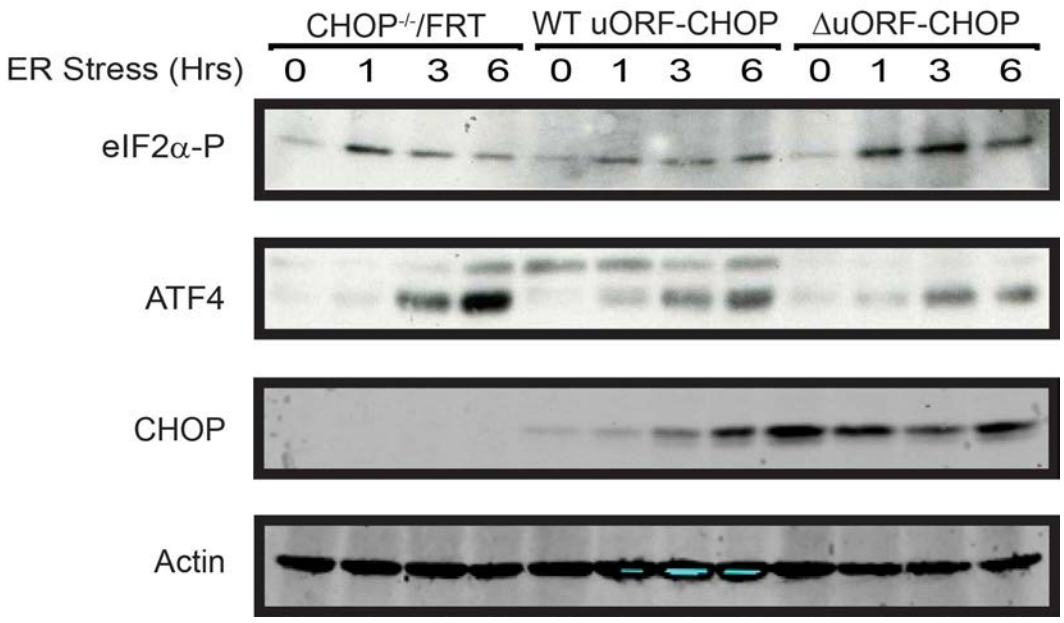


Figure 19. Deletion of the uORF in the *CHOP* mRNA leads to elevated expression of CHOP protein. CHOP$^{-/-}$/FRT cell lines that express *CHOP* encoding the 5'-leader with a WT uORF or deleted uORF were treated with 0.5 μM thapsigargin for up to 6 hours, or to no stress treatment (0). Lysates were prepared and the levels of eIF2~P, ATF4, CHOP, and β-actin were measured by immunoblot analysis using antibody that specifically recognizes each protein.

an increase in eIF2~P levels by 1 hour of thapsigargin treatment, which was sustained after 6 hours of treatment. ATF4 protein levels were higher in *CHOP*$^{-/-}$cells compared to WT-uORF-CHOP cells or ΔuORF-CHOP cells. As expected, CHOP levels were low in WT-uORF-CHOP cells after 1 hour of stress, but levels increased with time over the duration of the extended stress. These results support the model that the presence of uORF inhibits the CHOP protein synthesis at basal conditions. However, stress induced eIF2~P facilitates bypass of the inbibitory uORF, and thereby increases CHOP protein synthesis (Fig. 18). Compared to WT-uORF-CHOP cells, the ΔuORF-CHOP cells expressed high levels of CHOP protein at basal conditions. CHOP protein levels first decreased modestly during the initial hours of stress, but then increased by the later 6 hour treatment. Decrease in CHOP protein levels during the initial hours of stress could be attributed to repression of general translation mediated by eIF2~P. Interestingly, cells that express high CHOP protein have lower levels of ATF4 protein, suggesting that CHOP protein may represses *ATF4* expression through a feedback mechanism, such as for TRB3 (168).

8. Enhanced expression of *CHOP* sensitizes cells to apoptosis

What are the biological consequences if CHOP levels are enhanced in the absence of uORF? CHOP transcriptionally regulates gene expression initiating a proapototic response as a result of prolonged ER stress (86, 153). We hypothesize that an increased translation of *CHOP* from mRNA devoid of the uORF would sensitize the cells to apoptosis. To test this hypothesis, WT-uORF-CHOP or ΔuORF-CHOP cells were treated with thapsigargin for 15 hours or 20 hours, or no stress. Following the ER stress, cell

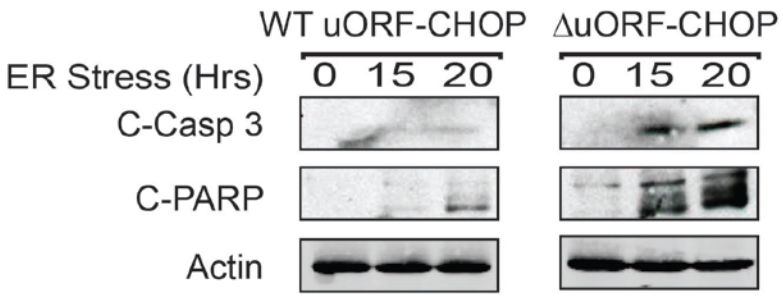
lysates were analyzed by immunoblot for the apoptosis markers, cleaved caspase-3 and PARP. WT-uORF-CHOP cells were found to have lower levels of cleaved caspase-3 and cleaved PARP compared to ΔuORF-CHOP cells (Fig. 20A), indicating that elevated CHOP levels sensitized the ΔuORF-CHOP cells to apoptosis during ER stress.

Cell viability assays confirmed that increased *CHOP* expression sensitized ΔuORF-CHOP cells cells to apoptosis during the prolonged ER stress (Fig. 20C). When cells were treated with the ER stress agent thapsigargin, about 55% of ΔuORF-CHOP cells were viable, whereas 70% of WT-uORF-CHOP cells were viable by 24 hours of stress. In cells treated with tunicamycin, another inducer of ER stress, similar results were found. About 50% of ΔuORF-CHOP cells and 70% of WT-uORF-CHOP cells were viable by 24 hours. These experiments support the a model where increases in CHOP levels due to the loss of the uORF sensitize cells to apoptosis in response to prolonged ER stress.

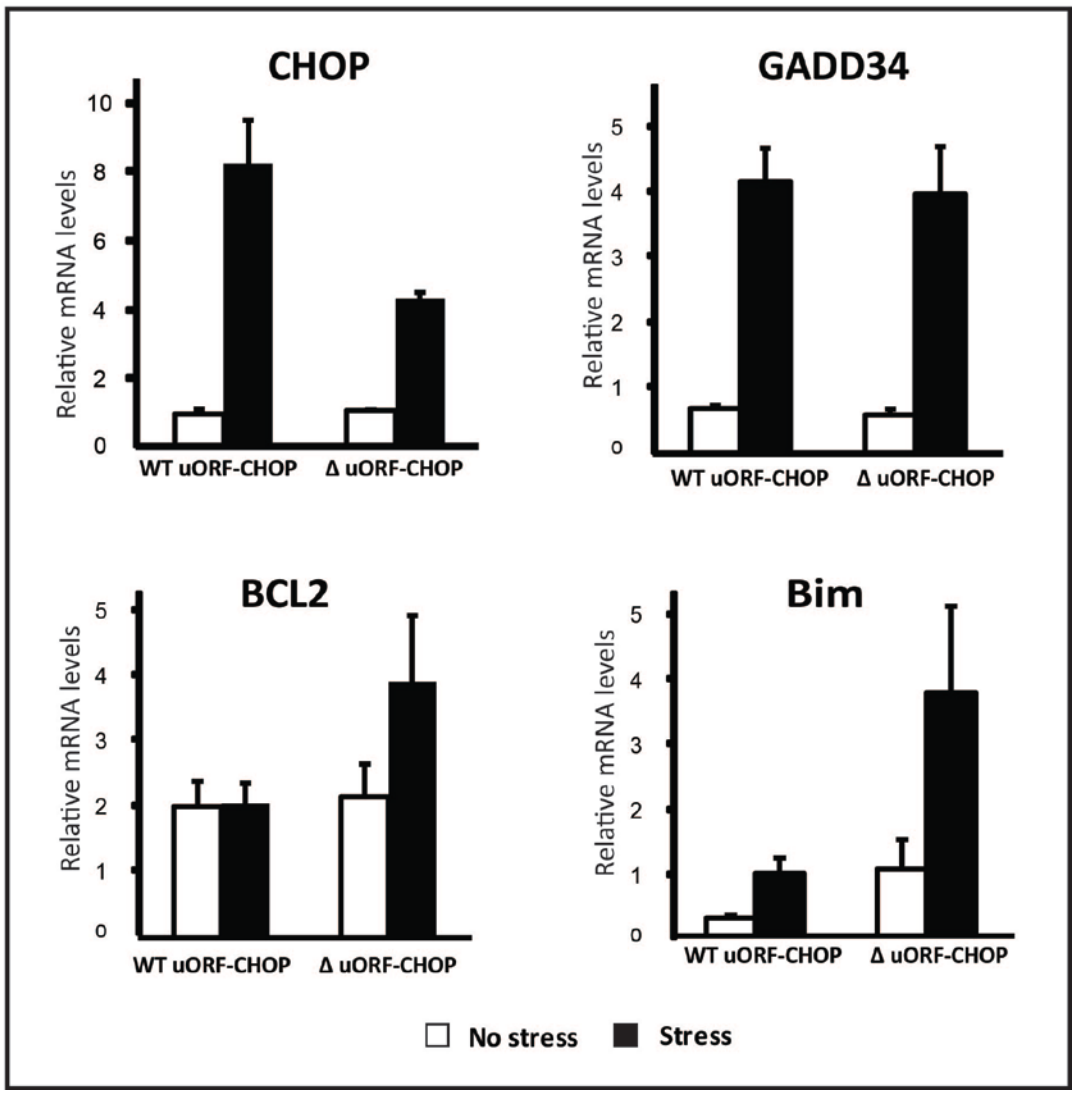
CHOP is suggested to enhance transcription of genes involved in the regulation of apoptosis. WT-uORF-CHOP or ΔuORF-CHOP cells were also treated with ER stress for 6 hours, or to no stress, and the mRNA levels of *CHOP*, *GADD34*, *BCL2* and *BIM* were measured (Fig. 20B). Consistent with previous observations, the WT-uORF-CHOP cells showed about a 20-fold increase in *CHOP* transcript levels in response to ER stress. However, *CHOP* mRNA levels in the ΔuORF-CHOP cells were induced only 10 fold higher than basal levels. Therefore, the overall increase in *CHOP* translation due to loss of the uORF is a significant contributor to the overall high levels of CHOP protein measured in the ΔuORF-CHOP cells.

CHOP increases *GADD34* transcription in response to ER stress (4, 31, 34). However, *GADD34* transcript levels were about the same in both WT-uORF-CHOP and ΔuORF-CHOP cells. This discrepancy may be due to decreases in ATF4 levels observed in the ΔuORF-CHOP cells, as ATF4 is also a transcriptional activator of *GADD34*. There was minimal change in *BCL2* transcript levels in cells with low *CHOP* expression. Surprisingly, *BCL2* transcript levels were increased by 50% with high *CHOP* expression in response to ER stress. Prior studies suggested that CHOP contributes to repression of *BCL2* (169). Importantly, the ΔuORF-CHOP cells showed a 3-fold increase in *BIM* transcript levels in response to stress compared to the WT-uORF-CHOP counterpart. CHOP enhancement of *BIM* transcription was reported to a significant contributor to increased apoptosis during ER stress (152), and this change in gene expression may be one important reason for the increased apoptosis observed in the ΔuORF-CHOP cells.

A)


WT uORF-CHOP
ΔuORF-CHOP
ER Stress (Hrs)
0 15 20
0 15 20
C-Casp 3
C-PARP
Actin

B)


CHOP
GADD34
BCL2
Bim
Relative mRNA levels
WT uORF-CHOP
Δ uORF-CHOP
No stress
Stress

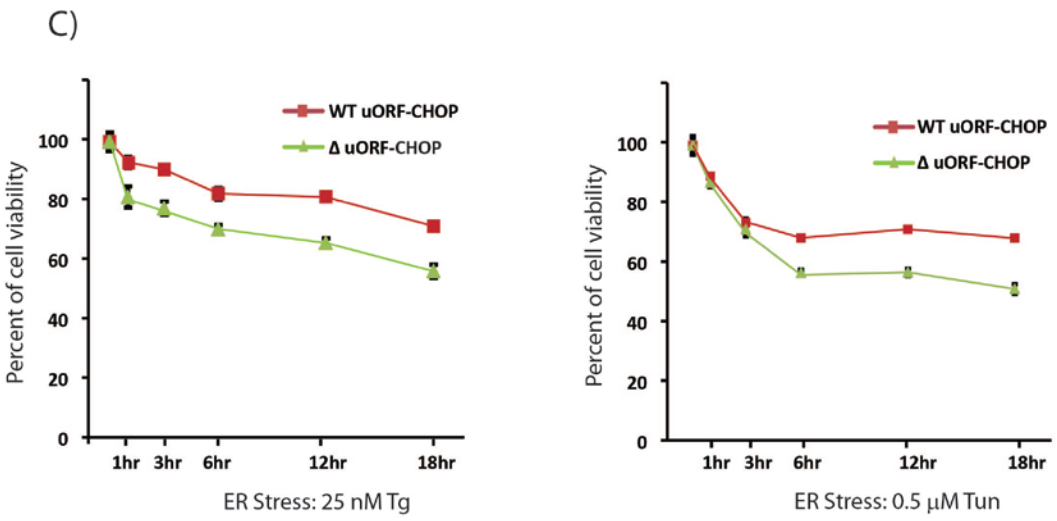


Figure 20. Enhanced expression of *CHOP* sensitizes cells to apoptosis in response to ER stress. (A) FRT recipient cell lines expressing WT-uORF-CHOP or ΔuORF-CHOP were treated with 0.5 μM thapsigargin for 15 hours and 20 hours, or no stress (0). Cell lysates were prepared and the levels of cleaved PARP and caspase-3, as well as β-actin were measured. (B) Total RNA was isolated from WT-uORF-CHOP or ΔuORF-CHOP cells treated with 0.5 μM thapsigargin for 6 hours (stress), or to no stress, and the relative levels of *CHOP*, *GADD34*, *BCL2*, and *BIM* mRNA were measured by qRT-PCR. (C) WT-uORF-CHOP or ΔuORF-CHOP cells were seeded in 96-well plates and allowed to adhere overnight. Cells were treated with ER stress agent 25 nM thapsigarin or 0.5 μM tunicamycin for up to 18 hours, or to no stress (0 hour), as indicated. The stress agent was then removed and then cells were incubated with culture medium in the absence of the stress agent for a total of 24 hours from the onset on the stress induction. Optical density representing viable cells was then determined by the CellTiter 96® Non-Radioactive Cell Proliferation Assay. Results are calculated as percentage of viable cells compared with control cultures that were not treated with stress (mean ± S.D., n = 3).

DISCUSSION

1. The uORF is central for regulation of *CHOP* translation in response to eIF2~P

eIF2~P inhibits general translation concurrent with preferential translation of select mRNAs, such as *ATF4, ATF5* and *CHOP*. Inhibition of global translation by eIF2~P can differentially lower translation of mRNAs genome-wide, with some gene transcripts being repressed, while others are more resistant to eIF2~P. This gradient model for translational repression suggests that the translational machinery can delineate between transcripts genome-wide to determine the degree of repression (Fig. 7). The degree of repression may involve a myriad of features in the 5'-leaders for each gene transcript, as well as possibly the 3'-untranslated regions (6). In this study, we addressed the underlying mechanisms by which eIF2~P can recalibrate the protein synthetic machinery, such that mRNAs are individually evaluated, leading to prescribed changes in their translation efficiencies. The 5'-leader of the *CHOP* mRNA has a single uORF, which is a significant barrier to *CHOP* translation in nonstressed conditions (Fig. 9). However, in response to ER stress, induced eIF2~P facilitates bypass of the repressing uORF, allowing scanning ribosomes to instead initiate translation at the *CHOP* coding region (Fig. 9).

Underlying this translation bypass mechanism is the initiating context of the *CHOP* uORF. The uORF has two AUGs, which are conserved at positions 1 and 4 throughout vertebrates (Fig. 11). Either AUG1 or AUG2 can serve as an initiation codon, although AUG2 is predominant, as viewed by the finding that loss of this second AUG in the uORF leads to the high expression of a *CHOP-Luc* reporter in the absence of stress, *i.e.* the greatest suppression of the inhibitory function of the uORF (Fig. 12). Central to

the ability of eIF2~P to direct the bypass of AUG1 and AUG2 is their less than optimal sequence context for translation initiation, a feature that is conserved among each of the mammalian orthologs of *CHOP* illustrated in Fig. 11B. Substitution of the Kozak consensus sequence for AUG2, in the absence of AUG1, significantly reduced the ability of ER stress and eIF2~P to overcome the inhibitory properties of the uORF (Fig. 14).

A second feature central to this model is the idea that translation of the uORF is suggested to block translation elongation or termination (Fig. 16). This idea was supported by the observation that the *uORF-Luc* fusion gene was poorly translated, with minimal expression of the fusion protein or luciferase activity (Fig. 16, *A* and *B*). Critical to this translation block is uORF residues 24–34, as deletion of this segment of the uORF allowed for translation of the fusion protein. When this region of the uORF was removed in the 5'-leader of the *PTK-CHOP-Luc* reporter, luciferase activity was significantly elevated during both stressed and nonstressed conditions (Fig. 16*C*). This was observed even when Δ24–34 was combined with a start codon containing the Kozak initiation context that was substituted into the uORF. In this case, eIF2~P would not facilitate bypass of the uORF, but the loss of this inhibitory segment is compromised, which could allow for some translation reinitiation to occur at the downstream *CHOP* coding region. The importance of the polypeptide sequence for the inhibitory function of the uORF is also supported by Jousse *et al.* (27), who first reported that the uORF can repress *CHOP* expression. Although the mechanism of alleviation of this inhibition was not addressed in this earlier study, it was found that the repressing properties of the uORF were significantly overcome by shortening of the uORF to three residues in length or by introducing a frameshift that alters the sequence but not the length of the encoded

polypeptide (Fig 17). The carboxy-terminal 11 amino acid residues (RRKCIFIHHHT), along with a methionine start codon, would be sufficient to confer repression properties to the uORF. Mutation of a 3 residue region (IFI) in the carboxy-terminal portion of the uORF encoded peptide is sufficient for loss of the uORF repression function (Fig. 17). Together these results support the model that translation of the carboxyl-terminal portion of the uORF polypeptide is critical for the repressing function of the uORF. Although the RNA sequence or structure *per se* does not appear to serve as a barrier to translation of the downstream *CHOP* coding region, it is possible that these RNA elements could serve as a contributing factor. The uORF is suggested to prevent ribosomal reinitiation at the downstream coding region in the mRNA and also potentially interfere with scanning of subsequent ribosomes proceeding from the 5'-end of the *CHOP* mRNA.

2. Translational control of *CHOP* and *ATF4* differ in fundamental ways

The mechanism of *CHOP* translational control is different from that described for *ATF4* in several fundamental ways. Although both *CHOP* and *ATF4* regulation involve uORFs programmed into the 5'-leaders of their mRNAs, the configuration of the uORFs and their function differ. *ATF4* and its yeast counterpart *GCN4* require two or more uORFs (1, 8, 11). The 5'-proximal uORF serves as a positive regulatory element that facilitates subsequent ribosome scanning and translation reinitiation at a downstream ORF. The levels of eIF2- GTP directed by induced eIF2~P determine how rapid this reinitiation event will be. During periods of no stress, when eIF2~P is reduced and eIF2-GTP is plentiful, ribosome reinitiation occurs rapidly at an adjacent inhibitory uORF, thus blocking expression of the transcription factor. In the case of *ATF4*, a single

inhibitory uORF is sufficient, whereas *GCN4* mRNA contains three short inhibitory uORFs, and translation of any one is thought to be sufficient to release ribosomes from the transcript. When eIF2~P is enhanced during stress, the resulting lowered eIF2-GTP levels delay translation reinitiation. As a result, reinitiating ribosomes can scan through the more immediate downstream inhibitory uORFs and instead initiate translation at the *ATF4* coding sequence.

Although the *CHOP* regulatory model shares with *ATF4* translational control the idea that eIF2~P can bypass an inhibitory uORF, it accomplishes this without the aid of a positive acting uORF that facilitates translation reinitiation. Instead *CHOP* has devised a single uORF with a poor translation initiation context that can be bypassed in response to eIF2~P (Fig. 18). In this way translational control induced by eIF2~P is no longer viewed as requiring two or more uORFs, but rather a single uORF in the specified context will suffice. eIF2~P induced by stress does not appear to significantly reduce the binding of the 43 S preinitiation complex (consisting of the 40 S ribosomal subunit and translation initiation factors including eIF2-GTP-Met-tRNAi Met) to the 5'-end of the *CHOP* mRNA. This is a feature shared with the *ATF4* and *GCN4* translational control models. Supporting this idea is the finding that the expression of *CHOP-Luc* deleted for both ATG1 and ATG2 was significantly elevated irrespective of stress conditions (Fig. 12). Furthermore, *CHOP-Luc* mRNA devoid of both initiation codons was broadly distributed among the fractions of the sucrose gradient during ER stress (Fig. 13). Rather eIF2~P is suggested to enhance the bypass of scanning ribosomes through an uORF with a poor initiation codon context.

We do not yet understand the biochemical basis for the eIF2~P bypass of the uORF in our model of *CHOP* translational control. Lowered eIF2-GTP levels may contribute to the reduced recognition of the *CHOP* uORF. Additional contributors to this bypass may be eIF2~P itself, or eIF2~P regulation of the expression of other critical translation factors, or regulators that would then facilitate the bypass of the *CHOP* uORF during stress conditions. It is noted that the translation factor eIF1 along with eIF2 and specific segments of 18 S rRNA are important for recognition of the initiation codon context (29, 37). It was reported that high levels of eIF1 can enhance the stringency for selection of gene start codons (38). However, our preliminary studies suggest that eIF2~P during ER stress does not significantly change the levels of eIF1 in MEF cells, with only a modest reduction during the early phase of thapsigargin treatment (Fig 15B). Given the proposal that eIF2~P can enhance bypass of gene coding regions with initiation codons with less than optimal initiation codon context, it is inviting to speculate that this feature may be a distinguishing feature between those mRNAs whose translations are severely repressed. By contrast, optimal Kozak context for initiation may contribute to enhanced translation resistance of gene transcripts during eIF2~P.

3. Role of *CHOP* translational control in stress responses

Expression of *CHOP* is central to the ISR and regulation of cell survival in response to environmental stress. Although the ISR can serve essential adaptive functions, chronic stress conditions and unabated expression of key target genes, such as *CHOP*, can contribute to morbidity (10, 22, 39). In this way, the ISR, which directs critical adaptive functions that can ameliorate cellular injury occurring during

environmental stresses, becomes maladaptive (Fig. 21). To study the role of the inhibitory uORF in vivo, we characterized the *CHOP*$^{-/-}$ cell line stably expressing *CHOP* transcripts containing a 5'-leaders with WT-uORF or aborted uORF (ATGs in uORF mutated to AGGs) (Fig. 19). Upon stress, the inhibitory uORF present in the 5'-leader of the CHOP mRNA served to tightly control the CHOP levels in response to eIF2~P. In wild-type cells, CHOP expression levels are low in non-stressed cells due to the uORF repression on *CHOP* translation. Stress-induced eIF2~P facilitates ribosome bypass of the inhibitory uORF and enhances CHOP levels. In the absence of the inhibitory uORF, the ΔuORF-CHOP cells expresses significantly higher levels of CHOP even in the absence of stress, which would mimic chronic stress induction of CHOP levels. Compared to WT-uORF-CHOP cells, the ΔuORF-CHOP cells have enhanced transcript levels of the apoptotic factor *BIM* during ER stress (Fig. 20A). Along with *BIM*, high levels of CHOP can direct the transcription of many other genes including *ERO1α, TRB3* which together can promote caspase activation and apoptosis (3, 17, 22, 40–42). Thus, the inhibitory uORF acts as a switch, which regulates CHOP protein levels in response to eIF2~P, shifting the cells from adaptive survival state in acute stress to apoptosis during chronic stress (Fig. 20C, 21).

In addition to transcriptional and translational regulation, *CHOP* mRNA and protein are subject to rapid turnover, with half-lives between 2 and 4 h (21). Therefore, the levels of CHOP protein are tightly linked to the current levels of stress insult and eIF2~P. The combination of transcriptional and translational regulation allows for a rapid increase in CHOP during ER stress, and with alleviation of the stress and diminished eIF2~P levels, *CHOP* translation levels are sharply reduced and *CHOP* mRNA and

protein subject to decay. This model emphasizes that CHOP levels are tightly linked to the amount of eIF2~P, which is adjusted rapidly to the extent of cellular stress. When a stress transcends from acute to chronic, these regulatory mechanisms would direct sustained elevated levels of CHOP protein and the products of its target genes, triggering apoptotic pathways (Fig. 21).

Mouse models deleted for the uORF of *C/EBPβ* (C/EBPβ$^{\Delta uORF}$) fail to initiate translation at *CEBPβ* LIP isoform resulting in hyper activation of acute phase genes, persistent repression of E2F-regulated genes, and impaired osteoclast differentiation (89). C/EBPβ$^{\Delta uORF}$ mouse models were the first created animals to demonstrate biological significance of translation control involving uORFs. Lee HC *et al*. (170), reported a transgenic zebra fish line called huORFZ that expresses human CHOP uORF fused to green fluorescent protein (GFP) encoding transcript, under CMV promoter driven transcription. Under normal conditions there was no GFP expression observed, supporting the idea that the uORF represses the downstream translation of the *CHOP* coding region. However, GFP expression could be observed only during stress conditions suggesting that stress facilitates bypass of inhibitory uORF to synthesize GFP. When these transgenic fish were exposed to various ER stress agents, prominent GFP expression was observed in various tissues depending on the stress agent, and on development stages (170). Though this model acts as reporter for translation control involving the CHOP inhibitory uORF, the influence of the uORF on stress-induced CHOP protein levels was not studied. A mouse model with a ΔuORF-CHOP mutation would be ideal for studying the full physiological ramifications of this translational control mechanism. This would allow for the study of the physiological impact of the

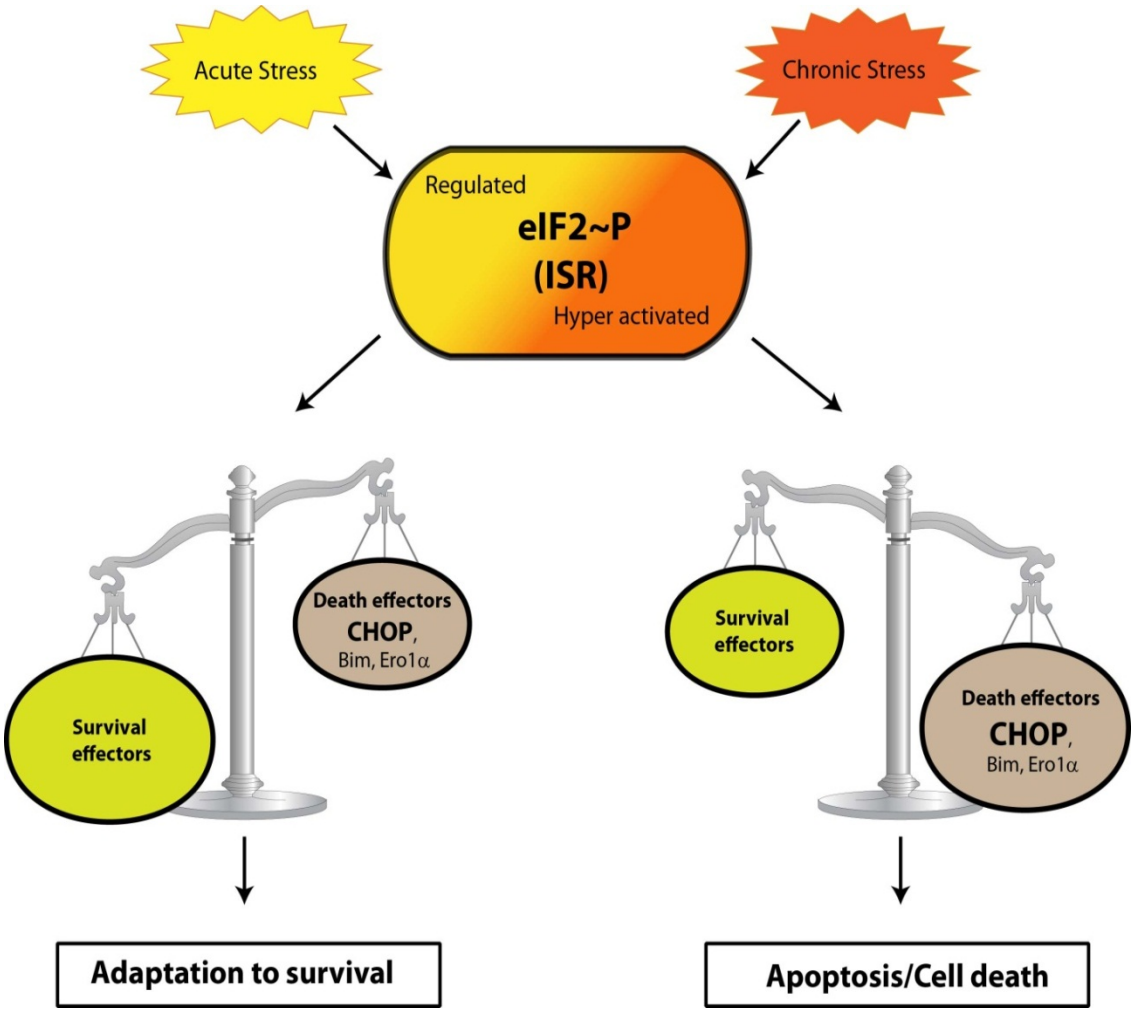


Figure 21. CHOP levels in response to stress induced eIF2~P are critical for cell fate. During variuous cellular stresses, eIF2 kinases phosphorylate eIF2α at serine 51. eIF2~P reduces global translation and induces preferential translation of mRNAs such as *ATF4* and *CHOP*, which are critical for ISR. Cells can adapt to acute stress conditions through eIF2~P induced ISR. Under non stressed conditions, the inhibitory uORF present in the *CHOP* mRNA serves to repress *CHOP* translation resulting in lower basal CHOP levels. During stress condition eIF2~P facilitates ribosome bypass of inhibitory uORF and increase CHOP levles. During chroninc stress, persistant eIF2~P and induced ISR

increases CHOP levels. The increase in CHOP protein shifts cells from adaptive survival to apoptosis by upregulating *BIM* and *ERO1α,* which are critical for initiating the apoptotic response.

inhibitory uORF on *CHOP* expression levels with its related pathologies and developmental changes.

4. Multiple mechanisms regulate *CHOP* expression and activity in response to stress

Here we report that CHOP protein levels may contribute to regulation of its own transcription. WT-uORF-CHOP cells showed some increased CHOP protein levels with elevated *CHOP* transcript levels (~9 folds) in response to ER stress compared to no stress. Whereas ΔuORF-CHOP cells have sharply elevated CHOP protein levels, with only partial increases in *CHOP* transcript levels in response to stress (Fig. 20A). It is noteworthy that MEF cells with over-expressed eIF1 have elevated CHOP protein levels, but reduced *CHOP* transcript levels in response to stress (Fig. 15B and C). These results, suggest a feedback mechanism by which CHOP can reduce its own transcription, perhaps indirectly by lowered activity of ATF4 (Fig. 19). This CHOP feedback mechanism may be yet another mode of regulation for maintaining CHOP expression in a small concentration range during acute stress. This is a topic that warrants further study.

Multiple stress pathways are thought to contribute to the levels of CHOP transcriptional activity during a given environmental stress. Chen *et al.* (20) suggested that, along with eIF2~P, *CHOP* translation can be regulated by phosphorylated eIF4E and its association with eIF4G. In this report, treatment with low concentrations of anisomycin increased *CHOP* mRNA levels and enhanced *CHOP* translation by a mechanism suggested to involve activation of the p38 MAPK/ MNK1 and mammalian target of rapamycin pathways. This alternative signaling scheme may also facilitate

CHOP translation by a process involving the uORF in the *CHOP* mRNA, suggesting that multiple signaling pathways regulated by stress may converge on *CHOP* translation. Phosphorylation of CHOP protein by p38 protein kinase has also been proposed to modulate its transcriptional activity, so this MAPK may contribute to regulation of CHOP function by multiple mechanisms (43). In the future, it will be important to determine whether these additional stress pathways function in conjunction with eIF2~P through the proposed bypass mechanism or entail alternative translational control processes involving the uORF or other features of the 5'-leader of the *CHOP* mRNA.

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PRESENTATIONS AND MEETINGS

1. **Palam LR**, Baird TD, Wek RC. Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance *CHOP* translation. Poster presented at 'Nutrient control of metabolism and Cell signaling' FASEB summer research conference, Steamboat Springs, CO, Aug 7^{th} -12^{th}, 2011
2. **Palam LR**, McClintick J, Liu Y, Wek RC. Genome-wide analysis of translational control induced in response to ER stress. Poster presentation, Biochemistry Research Day 2010, Indiana University School of Medicine

3. Babcock JT, **Palam LR**, He Y, Wek RC, Quillium LA. 4-Phenylbutyric acid: An AMPK agonist and mTORC1 inhibitor. Poster presentation, Biochemistry Research Day 2010, Indiana University School of Medicine
4. Zhou D, **Palam LR**, Staschke KA, Wek RC. Phosphorylation of eIF2 Directs ATF5 Translational Control in Response to Different Stresses. Presented at Cold Spring Harbor translational control meeting, Cold Spring Harbor, NY, September 3 –7, 2008
5. Molecular Medicine and Health Symposium, from February 23-24 2005, organized by Association for the Promotion of DNA Fingerprinting and other DNA Technologies, Center for Cellular & Molecular Biology, Hyderabad, India.
6. EMBO workshop on "Cell Interactions in Development and Disease", from Dec 16-18, 2004 held at Centre for Cellular & Molecular Biology, Hyderabad, India

PUBLICATIONS

1. Dai MS, Challagundla KB, Sun XX, **Palam LR**, Zeng SX, Wek RC, Lu H. (2012) Physical and functional interaction between ribosomal protein L11 and the tumor suppressor ARF. J. Biol. Chem. 2012 Mar 30. [Epub ahead of print]
2. **Palam LR**, Baird TD, Wek RC. (2011) Phosphorylation of eIF2 facilitates ribosomal bypass of an inhibitory upstream ORF to enhance *CHOP* translation. J. Biol. Chem. 286(13): 10939-10949
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